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Shaping of adaptive immune responses to soluble protein antigens by pathogen-associated molecular patterns

By

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Abstract

Invading organisms are detected by the innate immune system, through the recognition of conserved microbial structures. Innate responses are known to influence the development of adaptive immune responses, which are crucial for preventing infection and eliminating pathogens. Characterising the signals that initiate the induction of efficient cellular and humoral adaptive immune responses is particularly relevant for the rational design of new vaccines. The aims of this study were first to assess the ability of a broad range of conserved microbial stimuli to induce $CD8^+$ T cells responses by cross-priming and enhance antibody responses against exogenous soluble protein antigens, and secondly to investigate the mechanisms by which microbial stimuli induced cross-priming. Stimulation of Toll-like receptors (TLR) is believed to play a major role in the activation of innate and subsequent adaptive responses. All TLR agonists tested enhanced antigen-specific antibody responses, and in particular zymosan (TLR2/6), poly(I:C) (TLR3), LPS from *E. coli* (TLR4) and CpG DNA (TLR9) promoted IgG2a responses, which are thought to contribute effectively to protective mechanisms against pathogens in mice. However, only poly(I:C), LPS from *E. coli* and CpG DNA were able to stimulate the induction of cross-priming, whereas zymosan, peptidoglycan (TLR2/?) and R-848 (TLR7) were ineffective. It is known that LPS from different bacteria species can elicit different immune responses. LPSs from *Klebsiella pneumoniae* and from *Neisseria meningitidis*, but not the unconventional LPS from *Porphyromonas gingivalis*, were able to induce cross-priming. Microbial mannose structures, as present in yeast mannan, *Influenza* hemagglutinin and polymannose LPS, were demonstrated for the first time to be able to induce functional cross-priming. In addition, mannan and polymannose LPS were found to enhance antigen-specific antibody responses and promote IgG2a responses. IFN-α/β and signalling through costimulatory molecules play a central role in the licensing of cross-priming. Experiments using knock-out mice showed that in all cases licensing of cross-priming was dependent on IFN-α/βR signalling, albeit to a varying degree. In contrast, signalling through CD40 was not required for induction of cross-priming by mannan and polymannose LPS. Induction of cross-priming by LPS from *E. coli* and by mannan was TLR4-dependent, whereas induction by polymannose LPS was TLR4-independent.

This study thus identifies LPS from some bacteria species as cross-priming-inducing stimuli. It also confirms that activation of TLR can initiate induction of cross-priming, while indicating the existence of TLR-independent pathways. In addition, this work illustrates the importance of IFN-α/βR signalling as a cross-priming licensing stimulus.

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Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ANOVA	Analysis of variance
APC	Antigen presenting cell
BCR	B cell receptor
CARD	Caspase recruitment domain
CFA	Complete Freund's adjuvant
CGG	Chicken gamma globulin
CpG DNA	Cytidine-phosphate-guanosine deoxyribonucleic acid
CR	Complement receptor
CRD	Carbohydrate recognition domain
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing non-integrin
EBV	Epstein-Barr virus
EJIVR	Edward Jenner Institute for Vaccine Research
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain
FcγR	Fcγ receptor
GTPase	Guanosine triphosphatase
HA	Hemagglutinin
Hsp	Heat-shock protein
HSV	Herpes simplex virus
IAH	Institute for Animal Health
Ig	Immunoglobulin
IKK	IκB kinase
i.m.	Intramuscular
i.p.	intraperitoneal
IPS-1	IFN-β-promoter stimulator 1
IRAK	IL-1 receptor-associated protein kinase
IRF	IFN regulatory factor
i.v.	intravenous
LBP	Lipopolysaccharide-binding protein
LCMV	Lymphocytic choriomeningitis virus
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
MBL	Mannan-binding lectin
MD-2	Myeloid differentiation protein 2
mda-5	Melanoma differentiation-associated gene 5
MPL	Monophosphoryl lipid A
MR	Mannose receptor

NDV	Newcastle disease virus
NO	Nitric oxide
Nod	Nucleotide-binding oligomerisation domain
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PGRP	Peptidoglycan recognition protein
PKR	dsRNA-dependent protein kinase
poly(I:C)	polyinosinic:polycytidylic acid
RIG-1	Retinoic acid inducible gene 1
RIP	Receptor interacting protein
dsRNA	double-stranded ribonucleic acid
ssRNA	single-stranded ribonucleic acid
s.c.	subcutaneous
SCID	Severe combined immunodeficiency
SIGN-R	SIGN-related receptor
SP	Surfactant protein
SPF	Specific-pathogen free
SR	Scavenger receptor
SRCD	Scavenger receptor collagenous domain
TANK	TNF receptor-associated factor (TRAF)-family-member-associated NF-κB activator
TAP	Transporter associated with antigen presentation
TBK1	TANK-binding kinase 1
TCR	T cell receptor
Th cell	T helper cell
TICAM	TIR-containing adaptor molecule (TICAM-1 is also known as TRIF, and TICAM-2 as Tram)
TIR	Toll/IL-1 receptor homology domain
TIRAP	TIR domain-containing adaptor protein (also known as Mal)
TLR	Toll-like receptor
TRAF6	TNF receptor-associated factor 6
VSV	Vesicular stomatitis virus

Chapter 1

Introduction

The immune system is a coordinated body of specialised cells and soluble factors that cooperate to confer protection against harmful self and non-self.

Two divisions have evolved to form the immune system of vertebrates: the evolutionary conserved innate immune system, under the control of germline-encoded genes, and the adaptive immune system, characterised by somatic gene recombination and mutation. Pathogens that breach entry into the body are initially detected by the innate immune system, which is able to identify the nature of the pathogen and start eliminating it, while alerting the adaptive immune system and directing it for further appropriate defence mechanisms.

A particular type of innate cells, the dendritic cells (DC), is now known to play a pivotal role at the junction between the innate and adaptive immune systems. DC are able to integrate signals generated during the innate response and translate them into instructions for T cells and B cells, which activate effector functions accordingly. $CD4^+$ T cells coordinate the action of B cells and $CD8^+$ T cells, and provide feedback activation signals to innate effector cells.

$CD8^+$ T cells are the T cells in charge of killing infected cells. DC constantly present $CD8^+$ T cells with samples of intracellular contents. Infected DC themselves therefore present samples of invading pathogen to $CD8^+$ T cells. If $CD8^+$ T cells have a T cell receptor (TCR) specific for an antigen, and if the presenting DC has received information that the nature of the invader requires cell lysis for elimination, then specific $CD8^+$ T cells are primed to differentiate into cytotoxic T lymphocytes (CTL) and to lyse all cells bearing the specific antigen.

DC can also acquire pathogen samples from other infected cell types, which allows DC to initiate adaptive responses against pathogen that have no tropism for DC. Antigens acquired from the extracellular environment were traditionally thought to be presented exclusively to $CD4^+$ T cells. DC have the unique capacity to cross-present antigens acquired from other cells to $CD8^+$ T cells. However, similarly to priming of $CD8^+$ T cells against antigens synthesised within DC, full activation of

specific $CD8^+$ T cells against acquired antigens, which occurs by a process called cross-priming, is strictly controlled.

Cross-priming of $CD8^+$ T cells by DC needs to be authorised. Some microbial molecules have been found to allow cross-priming to occur, and a couple of signalling events, downstream of microbial recognition, have been described as cross-priming licensing stimuli.

It is important for adjuvant and vaccine development to define further which type of microbial molecules are able to stimulate authorisation of cross-priming and which licensing mechanisms may be involved in the generation of antigen-specific cytotoxic T cell responses. Current knowledge on how pathogens are detected, how the innate and adaptive immune systems are activated and what the adaptive response consist of, was taken into account for the design of this project.

1.1 Pathogen detection and activation of innate immunity

In order to explain peripheral tolerance of adaptive immune cells to normal self-antigens while considering adaptive cells requirement for costimulatory signals before clonal activation, Charles A. Janeway, Jr hypothesised that evolutionary primitive receptors controlled activation of both the innate and adaptive immune responses by stimulating effector functions only in response to infectious non-self molecules. Specificity to infectious non-self would be insured by multiple evolutionary selected innate receptors, each recognising a conserved structure common to a range of pathogens (Janeway, 1989; Janeway, 1992). These structures, targets of innate immune recognition, were termed pathogen-associated molecular patterns (PAMPs).

At least one soluble innate immune receptor, the mannan-binding lectin (MBL) (see paragraph 1.1.1.1.2) (Kuhlman *et al.*, 1989), able to bind specific pathogen structures, was already known. Within the following ten years though, the existence of evolutionary conserved receptors, able to recognise PAMPs and initiate specific intracellular signalling, was demonstrated in insect first, then in mammals (Lemaitre *et al.*, 1996; Medzhitov *et al.*, 1997; Rock *et al.*, 1998). Other receptors have been reported since. The major families of receptors involved in recognition of and responses to PAMPs are described below.

1.1.1 Innate immune receptors

1.1.1.1 Extracellular and membrane-bound receptors

1.1.1.1.1 Toll-like receptors

Toll-like receptors (TLR) are innate receptors homologous to the *Drosophila* Toll protein. *Drosophila* Toll was identified as a component of a signalling pathway controlling dorsoventral polarity in the fly embryo (Hashimoto *et al.*, 1988). Toll was found to have a cytoplasmic domain that is related to human interleukin-1 receptor (IL-1R) (Gay *et al.*, 1991), which can induce translocation of NF-κB, a gene transcription factor for a wide variety of immune response genes, to the nucleus. *Drosophila* Toll pathway was later found to be involved in the specific expression of antifungal immune response in the fly, since inactivation of *toll* gene resulted in high susceptibility to fungal infection but not to bacterial infection (Lemaitre *et al.*, 1996). Other members of the Toll family, along with more recently described receptors, such as peptidoglycan recognition proteins (PGRP), enable *Drosophila* to discriminate between classes of pathogens (fungi, Gram-positive and Gram-negative bacteria) and activate the secretion of killing peptides with appropriate activity against the structure of the pathogen (Tauszig *et al.*, 2000; Leclerc *et al.*, 2004).

Shortly after the contribution of Toll to *Drosophila* immunity was described, a family of human homologues of Toll was identified and termed Toll-like receptors (Rock *et al.*, 1998). Since then, the protein sequences of 13 mammalian TLR have been described (Tabeta *et al.*, 2004), and the specificity of most of them has been characterised (Akira, 2003; Takeda *et al.*, 2003; Kawai *et al.*, 2005a).

TLR are membrane-bound glycoproteins that are present either at the cell surface or sequestered in intracellular compartments (endosome). Their agonist recognition domain faces outside the cell or the lumen of the endosome. TLR recognition domain contains leucine-rich repeats (LRR) (Bell *et al.*, 2003).

The first TLR to be characterised was TLR4. First, a constitutively active mutant was constructed, and transfected cells were found to be induced to express genes coding for cytokines and costimulatory molecules, as well as to activate NF-κB, indicating that this TLR had the capacity to activate innate immune signalling pathways that can lead to innate responses that support the generation of adaptive responses (Medzhitov *et al.*, 1997). Importantly, the *tlr4* gene was assigned to a chromosomal

region that corresponded to the location of the *Lps* gene, where the mutation responsible for hyporesponsiveness to endotoxin in C3H/HeJ mice was mapped (Watson *et al.*, 1978). After cloning of mouse *tlr4*, the exact mutation responsible for the phenotype of both C3H/HeJ and C57Bl/10ScCr mice was characterised (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). The generation and study of TLR4-deficient mice confirmed that the release of pro-inflammatory cytokines and B cell proliferation in response to LPS from *E. coli* and *S. minnesota* depended on TLR4 (Hoshino *et al.*, 1999). TLR4 activity is not restricted to bacterial LPS, since recently TLR4 was shown to be involved in responses to fungal and viral components (Tada *et al.*, 2002; Jiang *et al.*, 2005).

TLR4 does not act alone. LPS is first bound by a serum protein, lipopolysaccharide binding protein (LBP), that binds the lipid A moiety of LPS (Tobias *et al.*, 1989), helps disrupting LPS micelles and transfers LPS monomers to CD14 to form a complex (Schumann *et al.*, 1990; Wright *et al.*, 1990; Gioannini *et al.*, 2004). CD14-LPS then binds myeloid differentiation protein 2 (MD-2), a glycoprotein that interacts with TLR4 LRR domain and that is essential in LPS recognition (Schromm *et al.*, 2001; Nagai *et al.*, 2002). Signalling through TLR4 is probably stimulated by TLR dimerisation and change in conformation, which may be influenced by CD14 and/or MD-2 (Gangloff *et al.*, 2004; Saitoh *et al.*, 2004; Kim *et al.*, 2005a). Induction of CD14-dependent and -independent immune responses to LPS has been reported (Perera *et al.*, 1997; Hamann *et al.*, 2005; Jiang *et al.*, 2005).

CD14 is also a co-receptor for some of TLR2-mediated responses. TLR2 responds to PAMPs from various organisms, including from Gram-negative bacteria (porins from *Neisseria meningitidis* (Massari *et al.*, 2002), LPS from some non-enterobacterial species (Tanamoto *et al.*, 1997; Werts *et al.*, 2001; Erridge *et al.*, 2004)), from Gram-positive bacteria (lipoteichoic acid (LTA) (Henneke *et al.*, 2005)) and from yeast (zymosan (Underhill *et al.*, 1999)). TLR2 has been reported to form heterodimers with at least TLR1 or TLR6, which allows differential immune responses depending on agonists. Triacylated lipopeptides and lipoproteins, such as synthetic bacterial structure Pam_3CSK_4 and *Mycobacterium tuberculosis* 19kDa lipoprotein, are detected by TLR2/TLR1, while diacylated lipopeptides Pam_2CSK_4 and MALP-2 are detected by TLR2/TLR6 (Takeuchi *et al.*, 2002). CD14 has been reported to participate in TLR2/TLR1-mediated binding of lipopeptides (Vasselon *et*

al., 2004; Manukyan *et al.*, 2005), and TNF-α secretion in response to diacylated lipopeptide Pam_2CSK_4 (TLR2/6) has been shown to be partially CD14-dependent (Jiang *et al.*, 2005). As TLR1 and TLR6, TLR10 (Chuang *et al.*, 2001) is structurally related to TLR2, and it has been suggested it may form heterodimers with TLR2 (Akira, 2003).

Responses to protein PAMPs that may have in common a role in pathogen mobility and invasion have been found to be mediated by TLR5 (for bacterial flagellin (Hayashi *et al.*, 2001)) and murine TLR11 (for protozoal profilin (Yarovinsky *et al.*, 2005)).

Although closely related to TLR5, TLR3 mediates responses to a different category of PAMPs. Indeed, double-stranded RNA (dsRNA), generated as a synthesis intermediate or a transcription by-product during viral replication, can be recognised by TLR3 (Alexopoulou *et al.*, 2001).

TLR7 and human TLR8 detect guanosine- and uridine-rich single-stranded RNA (ssRNA) (Diebold *et al.*, 2004; Heil *et al.*, 2004), as well as synthetic imidazoquinolines such as imiquimod (R-837) and resiquimod (R-848) (Hemmi *et al.*, 2002; Jurk *et al.*, 2002).

TLR9 recognises unmethylated deoxyribonucleotide sequences containing cytidine-phosphate-guanosine motifs (CpG DNA) from bacteria and viruses (Hemmi *et al.*, 2000; Krieg, 2002; Abe *et al.*, 2005). TLR9 was recently shown to respond to a non-DNA agonist, hemozoin, which is a product from malaria's catabolism of host haemoglobin (Coban *et al.*, 2005).

In contrast to other TLR expressed at the cell surface on antigen presenting cells, TLR3, TLR7/8 and TLR9 are localised primarily in endosomes, where their recognition domain samples the inside of the endosome (Matsumoto *et al.*, 2003; Latz *et al.*, 2004a; Latz *et al.*, 2004b; Nishiya *et al.*, 2004).

Recognition and response to some PAMPs may be mediated by TLR in collaboration with other innate receptors, including some members of the lectin family (Mukhopadhyay *et al.*, 2004).

1.1.1.1.2 Lectins

Lectins are non-enzymatic proteins that can bind carbohydrates. C-type lectins in particular possess at least one carbohydrate recognition domain (CRD) that requires

calcium to interact with its ligand. Since many "lectins" structurally related to C-type lectins have CRD that function without calcium, these lectins are often collectively referred to as C-type lectin-like receptors, and their CRD are called C-type lectin-like domains. Many C-type and C-type lectin-like receptors are involved in immune processes (Cambi *et al.*, 2003a; Rogers *et al.*, 2003; McGreal *et al.*, 2004).

Dectin-1 was the first C-type lectin-like receptor reported to collaborate with a TLR (TLR2) for stimulation of cytokine and chemokine production in response to yeast zymosan (Brown *et al.*, 2003; Gantner *et al.*, 2003a). Dectin-1 actually recognises the β-glucan moiety of zymosan, and soluble and particulate β-glucans in general (Brown *et al.*, 2001; Brown *et al.*, 2005), and can also trigger cytokine production independently of TLR through its own cytoplasmic ITAM-like signalling domain (Rogers *et al.*, 2005).

Dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing non-integrin (DC-SIGN) and SIGN-related (SIGN-R) receptors (Geijtenbeek *et al.*, 2000; Park *et al.*, 2001; Geijtenbeek *et al.*, 2004) are C-type lectins that recognise high-mannose structures, such as presented by viral envelope glycoprotein gp120 from HIV, by mannose-capped lipoarabinomannan from *M. tuberculosis*, or by yeast mannan (Tailleux *et al.*, 2003; Takahara *et al.*, 2004; Snyder *et al.*, 2005). Specificity of DC-SIGN and SIGN-R homologues for high-mannose structures is achieved by multiple oligomerisation, forming tetramers of monomers, and clustering (Feinberg *et al.*, 2001; Mitchell *et al.*, 2001; Cambi *et al.*, 2005a; Cambi *et al.*, 2005b). The function of these C-type lectins is being debated, but recent reports support a role for DC-SIGN (Snyder *et al.*, 2005; Tacken *et al.*, 2005) and marginal zone macrophage SIGN-R1 (Kang *et al.*, 2003) in endocytosis and antigen presentation (Engering *et al.*, 2002; Cambi *et al.*, 2003b). On peritoneal macrophages, SIGNR-1 has been shown to collaborate with dectin-1 for the detection of zymosan, which contains β-glucan and mannan, although cytokine production in response to zymosan was independent of SIGNR-1 (Taylor *et al.*, 2004), consistent with the fact that SIGNR-1 and others members of this group have no obvious signalling motif. There is evidence however that recognition of specific PAMPs by DC-SIGN and SIGN-R promote stimulation of signalling pathways and immune responses (Geijtenbeek *et al.*, 2003; Bergman *et al.*, 2004), possibly by collaborating with TLRs, such as TLR4 (Nagaoka *et al.*, 2005).

The mannose receptor (MR) is the best characterised C-type lectin, mediating phagocytosis and endocytosis (Kruskal *et al.*, 1992; Sallusto *et al.*, 1995; Stahl *et al.*, 1998) of molecules bearing repeated polymers of mannose (Mullin *et al.*, 1994), such as displayed by some bacteria, viruses and yeasts (Milone *et al.*, 1998; Stehle *et al.*, 2000; Cinco *et al.*, 2001; Zamze *et al.*, 2002; Nguyen *et al.*, 2003; Porcaro *et al.*, 2003). A role for MR in antigen presentation has also been reported (Martinez-Pomares *et al.*, 1996; Prigozy *et al.*, 1997). In contrast, the precise contribution of MR to induction of immune responses has not been as well characterised. MR was shown however to be involved in mannose-dependent cytokine secretion in response to some viruses and to *Candida albicans* (Yamamoto *et al.*, 1997; Milone *et al.*, 1998). A probable collaboration with another innate receptor, possessing an intracellular signalling domain, has not been described.

DEC-205 (Jiang *et al.*, 1995) is structurally related to the MR and has therefore been classed as a member of the mannose receptor family (East *et al.*, 2002); carbohydrate binding activity or ligand specificity have still not been defined. DEC-205-specific antibodies have been used to target the receptor and it has been confirmed that DEC-205, which has an internalisation cytoplasmic tail motif, is an endocytic receptor (Mahnke *et al.*, 2000). In addition, DEC-205 possesses another motif that enables it to target ligands to late endosome and lysosome compartments where processing for antigen presentation takes place (Mahnke *et al.*, 2000). Targeting antigens to DEC-205 has been found to result in efficient MHC I and MHC II antigen presentation (Mahnke *et al.*, 2000; Bonifaz *et al.*, 2002).

Specificity for mannose and polymannose structures, such as displayed by DC-SIGN/SIGNR and MR, is characteristic of many other C-type lectins, including members of the collectin family (Hoppe *et al.*, 1994). Mannan-binding lectin (MBL) is an evolutionary conserved molecule that binds PAMPs and activates the complement proteolytic pathway (Hoffmann *et al.*, 1999; Teillet *et al.*, 2005). MBL recognises specific mannose structures on bacteria (Devyatyarova-Johnson *et al.*, 2000; Swierzko *et al.*, 2003; Lynch *et al.*, 2004), viruses (Gadjeva *et al.*, 2004; Botos *et al.*, 2005; Ji *et al.*, 2005) and fungi (Neth *et al.*, 2000; Pellis *et al.*, 2005), through oligomerisation of three monomers into trimers, which insures a spatial configuration appropriate for PAMP binding (Wallis, 2003). Two surfactant proteins (SP), SP-A and SP-D, also belonging to the collectin family, are not able to activate the

complement system, but bind various carbohydrate structures and are involved in innate responses to many pathogens (Hickling *et al.*, 2004; Palaniyar *et al.*, 2004). Complement receptor 1 (CR1) and the chaperone protein calreticulin are among the candidate receptors for SP-A and SP-D, but the role of SP-A and SP-D in immune responses will be better defined once their receptor(s) has been identified (Hickling *et al.*, 2004).

1.1.1.1.3 Complement proteins

C-reactive protein (CRP) is an acute phase serum protein that belongs to the evolutionary conserved family of pentraxin proteins (Du Clos *et al.*, 2003). CRP binds bacterial phosphocholine in particular, and phosphorylated carbohydrates (Volanakis *et al.*, 1971; Culley *et al.*, 2000; Lysenko *et al.*, 2000). CRP, upon PAMP binding, primarily activates the complement system by binding complement protein C1q. CRP is also able to stimulate intracellular signalling, through Fcγ receptor (FcγR) II, and innate cytokine responses (Ballou *et al.*, 1992; Galve-de Rochemonteix *et al.*, 1993; Chi *et al.*, 2002).

Complement protein C1q, like MBL, is often classed as a defence collagen. It has a collagen-like domain and globular domains that are able to bind polyanionic molecules such as bacterial LPS, as well as membrane components from bacteria, viruses and fungi, although precise PAMPs have not been characterised (Gasque, 2004). C1q may share its receptor, mediating phagocytosis and/or signalling activities, with SP-A and SP-D (Gasque, 2004; Hickling *et al.*, 2004). Complement protein C3 can also be activated on the surface of pathogens by binding carbohydrates and proteins. PAMPs-bearing pathogens can then be targeted to the complement receptor 3 (CR3, CD11b/CD18). CR3 also possess a carbohydrate binding site (Forsyth *et al.*, 1998; Brown *et al.*, 2005). CR3 is able to recognise β-glucan (Thornton *et al.*, 1996; Noubir *et al.*, 2004) and LPS, and is thought to cooperate with other receptors that would mediate activation of immune responses (Ehlers, 2000). CR3 for instance cooperates with CD14 to stimulate intracellular immune signalling (Medvedev *et al.*, 1998), possibly through triggering of a TLR. CR3 is also indispensable to FcγR III-mediated induction of cytotoxicity (Kushner *et al.*, 1992; van Spriel *et al.*, 2001).

1.1.1.1.4 Scavenger receptors

Scavenger receptors (SR) have been shown to be able to recognise PAMPs through their collagenous domain or their cysteine-rich domain (SRCD) (Linehan *et al.*, 2000; Taylor *et al.*, 2005). SR-A binds diverse polyanionic ligands. It can recognise lipid A from Gram-negative bacteria and LTA from Gram-positive bacteria (Dunne *et al.*, 1994). SR-A was also found to bind poly-G sequences and may play a role in helping the stimulation of cytokine production by A-class CpG DNA sequences, which is mediated by TLR9 (Lee *et al.*, 2000). CD36 is a different type of SR, which has no collagenous domain. It is a conserved receptor (Franc *et al.*, 1996), and is able to recognise diacyl lipopeptides LTA and MALP-2, and contribute to responses induced through TLR2/TLR6 (Hoebe *et al.*, 2005; Stuart *et al.*, 2005).

In contrast to the membrane bound or soluble receptors described above, some receptors involved in PAMPs recognition and in initiation of immune responses are present in the cytoplasm.

1.1.1.2 Cytosolic receptors

Evolutionary conserved receptors with LRR domains, other than TLRs, can detect PAMPs. Nucleotide-binding oligomerisation domain (Nod) proteins Nod1 and Nod2 can mediate activation of signalling pathways in response to LPS (Inohara *et al.*, 2001; Ogura *et al.*, 2001). Both Nod1 and Nod2 recognise peptidoglycan, although each receptor specifically detects distinct motifs (Philpott *et al.*, 2004). Nod1 senses peptidoglycan from Gram-negative bacteria, while Nod2 recognises muramyl dipeptide present in peptidoglycan from both Gram-negative and Gram-positive bacteria. Nod2 was shown to be involved in controlling Th1 cytokine production in response to peptidoglycan and muropeptides (Pauleau *et al.*, 2003; Watanabe *et al.*, 2004; Fritz *et al.*, 2005).

A few IFN-inducible proteins present in the cytoplasm have been shown to bind dsRNA and activate signalling pathways. RNA helicase-containing proteins RIG-1 and mda-5 recognise intracellular synthetic dsRNA poly(I:C) and dsRNA-producing viruses (Andrejeva *et al.*, 2004; Yoneyama *et al.*, 2004). dsRNA is also recognised by protein kinase PKR, which signals for cytokine production (Balachandran *et al.*, 2000; Diebold *et al.*, 2003).

The immune system has hence evolved to detect pathogens, through the recognition of conserved structures by many innate receptors. Membrane-bound and soluble receptors make use of a variety of binding domains, including LRR, C-type lectin-like CRD, cysteine-rich and RNA binding domains, to collectively recognise PAMPs from all classes of pathogens, such as bacteria, viruses, fungi and parasites. Individual receptors however have defined specificities, and the immune system has evolved to gain further specificity through various combinations of cooperating receptors. Combinational recognition of a PAMP, multiplied by the however many PAMPs displayed by a pathogen, allows the immune system to initiate responses that, with evolutionary experience, proved to be appropriate to the class/species/strain of the PAMPs-bearing pathogen.

Differential innate immune responses are actually initiated by the triggering of the innate receptors that possess a domain capable of transducing a signal, in the specific context of other receptors that may form the PAMP-binding complex. The innate immune signalling pathways that are activated by PAMPs are being studied extensively, to try to decipher the codes for induction of immune responses to different pathogens. Current understanding of signalling pathways activated by PAMPs is summarised below.

1.1.2 Activation of innate immune genes

TLRs are a major family of signalling innate receptors, and results from numerous studies have enabled the characterisation of various intracellular events that take place when specific PAMPs activate individual TLRs.

TLRs, IL-1 receptors (IL-1R) and IL-1R-related receptors form a superfamily, which is defined by a homologous signalling domain called the Toll/IL-1R homology domain (TIR) (Gay *et al.*, 1991; Rock *et al.*, 1998; O'Neill *et al.*, 2000). Notably, MyD88, a member of the TLR/IL1-R superfamily that lacks an extracellular domain, was found to play an important role in transducing signal from IL-1R (Wesche *et al.*, 1997). The role of MyD88 was studied in the context of TLR signalling, and the contribution of other TIR-containing molecules as TLR adaptor molecules was investigated and characterised.

1.1.2.1 The TLR/MyD88 pathway

Homologies in the TIR domain of IL-1R and TLR suggested that, by analogy, TLRs may also use MyD88 as an adaptor molecule to transduce signals to the nucleus. Indeed, at least TLR2, TLR4, TLR5, TLR7/8, TLR9 and TLR11 have been shown to activate NF-κB and subsequent expression of inflammatory cytokines through MyD88 (Kawai *et al.*, 1999; Akira *et al.*, 2004; Coban *et al.*, 2005; Yarovinsky *et al.*, 2005). In contrast, TLR3 does not share the MyD88 pathway (Akira *et al.*, 2004).

TLR stimulation by ligation of PAMP and accessory receptors (Kennedy *et al.*, 2004; Vasselon *et al.*, 2004; Jiang *et al.*, 2005) promotes the recruitment of MyD88 to the TLR cytoplasmic TIR domain (Akira *et al.*, 2004). When MyD88 and TLR TIR domains interact, MyD88, which contains a death domain (DD), can interact with the DD of a serine/threonine protein kinase (IL-1R associated protein kinase, IRAK). In the case of TLR2 and TLR4 signalling, another TIR-containing molecule, TIR domain-containing adaptor protein (TIRAP), also known as Mal, binds to TLR2 and TLR4 and contributes to MyD88 signalling (Horng *et al.*, 2001; Yamamoto *et al.*, 2002a). MyD88 interaction with IRAK leads to a kinase signalling cascade. IRAK4 in particular (Suzuki *et al.*, 2002) autophosphorylates and activates IRAK1, which dissociates from the receptor complex and associates with TNF-receptor associated factor 6 (TRAF6) (Jiang *et al.*, 2002). TRAF6 transmits the phosphorylation signal to the IκB kinase (IKK) complex, which in turn activates the transcription factor NF-κB (Nomura *et al.*, 2000). NF-κB induces expression of inflammatory cytokines and chemokines (Collart *et al.*, 1990; Hiscott *et al.*, 1993; Garoufalis *et al.*, 1994; Hoffmann *et al.*, 2005; Osterlund *et al.*, 2005). The MyD88-TRAF6 signalling pathway can also activate the transcription factor IFN regulatory factor 5 (IRF5), which induces the expression of inflammatory cytokines (Barnes *et al.*, 2002a; Barnes *et al.*, 2004; Takaoka *et al.*, 2005). TLR7/8 induce IRF5-mediated production of IFN-α/β (Schoenemeyer *et al.*, 2005). TLR7/8 and TLR9 can additionally activate IRF7, which regulates the expression of IFN-α/β (Honda *et al.*, 2004; Kawai *et al.*, 2004; Honda *et al.*, 2005b; Schoenemeyer *et al.*, 2005; Uematsu *et al.*, 2005).

MyD88-independent TLR-mediated responses have been described (Kawai *et al.*, 1999; Alexopoulou *et al.*, 2001; Kawai *et al.*, 2001), and the adaptor molecules mediating those responses have been characterised.

1.1.2.2 The TLR/TRIF pathway

TLR3 and TLR4 can activate MyD88-independent signalling pathways (Kawai *et al.*, 2005a). It has been found that TIR-containing adaptor molecule TRIF (also known as TICAM-1) mediates TLR3-induced NF-κB and IRF3 activation (Yamamoto *et al.*, 2002b; Hoebe *et al.*, 2003a; Oshiumi *et al.*, 2003a); IRF3 is a nuclear transcription factor that induces the expression of IFN-β (Taniguchi *et al.*, 2001). TICAM-2, also known as Tram, a TIR-containing molecule homologous to TRIF, was shown to form a heterodimer with TRIF to bind TLR4 and mediate activation of IRF3 and NF-κB (Fitzgerald *et al.*, 2003b; Oshiumi *et al.*, 2003b; Yamamoto *et al.*, 2003; Cusson-Hermance *et al.*, 2005).

IRF3 activation is likely to occur through TRIF-induced phosphorylation of TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) and IKKε (Fitzgerald *et al.*, 2003a; Sharma *et al.*, 2003; McWhirter *et al.*, 2004). TLR3-dependent and TLR4-dependent activation of NF-κB involves phosphorylation of TRAF6 and receptor-interacting protein-1 (RIP1) (Sato *et al.*, 2003; Meylan *et al.*, 2004; Cusson-Hermance *et al.*, 2005). RIP2 may contribute to TLR4-activated signalling (Chin *et al.*, 2002; Kobayashi *et al.*, 2002; Lu *et al.*, 2005), possibly through a cross-talk between TLR4 and the Nod signalsome.

Indeed, RIP2 interacts with both Nod1 and Nod2 and mediates the activation of NF-κB (Girardin *et al.*, 2001; Kobayashi *et al.*, 2002).

Intracytoplasmic PRR RIG-1 probably activates IRF3 and NF-κB through signals transduced by its caspase recruitment domain (CARD) (Yoneyama *et al.*, 2004). A new adaptor, termed IFN-β promoter stimulator (IPS-1), has been shown to contain a CARD homologous to RIG-1 and mda-5 domains, and to interact with them (Kawai *et al.*, 2005b). IPS-1 was found to mediate activation of NF-κB and IL-8 production, through RIP1 and Fas-associated death domain (FADD) (Balachandran *et al.*, 2004; Kawai *et al.*, 2005b), and to activate IRF3-IRF7 and IFN-α/β expression, through TBK1 and IKKε.

The TLR/MyD88 and TRIF pathways are represented in Figure 1.1.

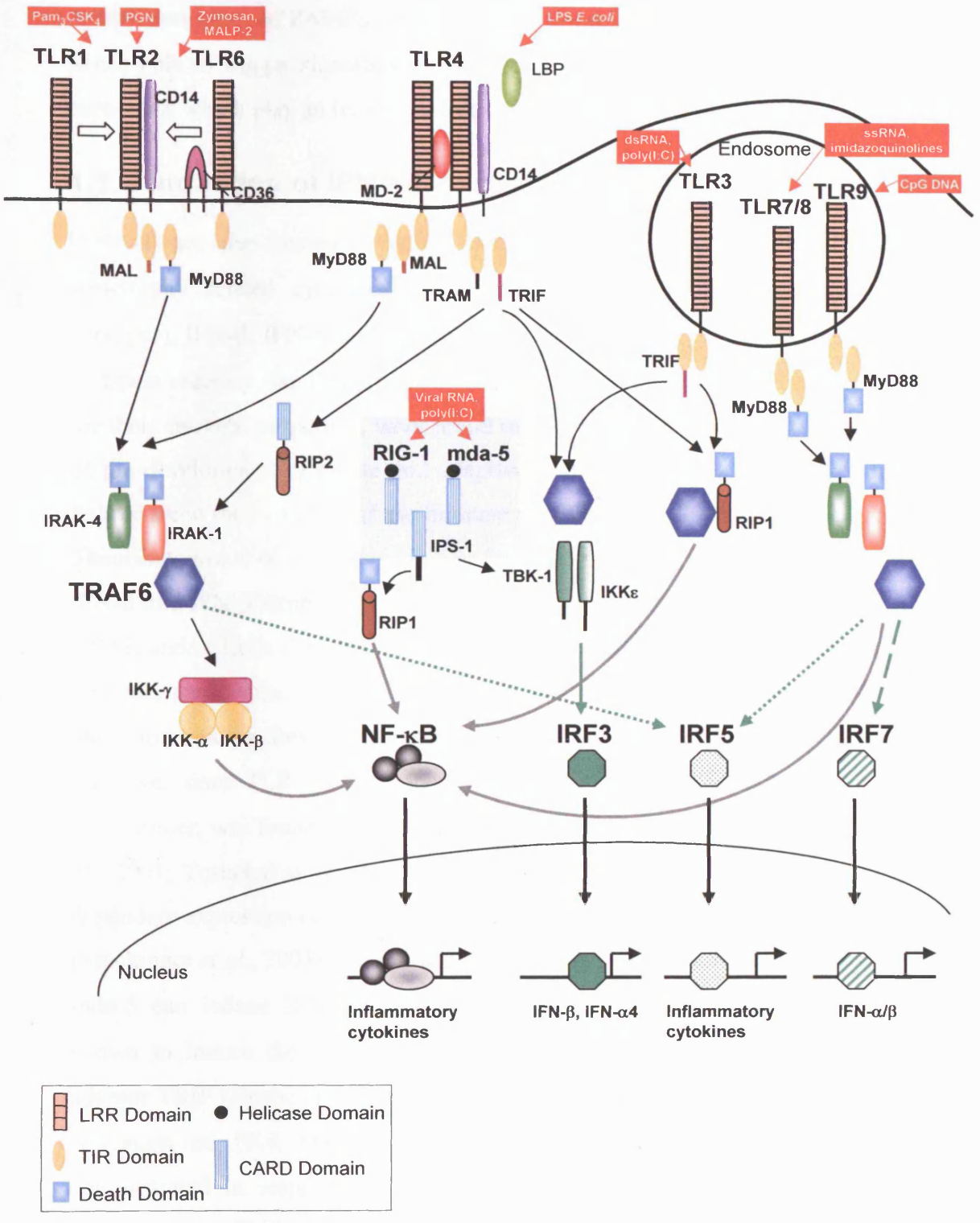

Figure 1.1. MyD88 and TRIF signalling pathways of Toll-like receptors.
NF-κB and/or IRF3, IRF5 and IRF7 signalling pathways of TLR2, TLR3, TLR4, TLR7/8, TLR9, and cytoplasmic helicases RIG-1 and mda-5, that control the expression of major immunostimulatory cytokines. Adapted from (Akira *et al.*, 2004; Kawai *et al.*, 2005a) and from reports referenced in 1.1.2.1 and 1.1.2.2.

A few categories of PAMPs, by stimulating certain TLR or cytolosic receptors, are hence able to trigger signalling cascades that control the expression of IFN-α/β in particular, which play an important role in promoting adaptive immune responses.

1.1.3 Induction of IFN-α/β expression

IFN-α/β are also known as type I interferons and belong to a family of many structurally related cytokines, which comprises IFN-α (subdivided into many subtypes), IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ, IFN-ω; all type I IFN bind a unique common receptor, the IFN-α/β-R (Pestka *et al.*, 2004). IFN-α/β, first characterised for their antiviral properties, were found to play an important role in various aspects of the development of innate and adaptive immune reponses, thereby serving as a link between the two arms of the immune system (Biron, 2001; Le Bon *et al.*, 2002; Theofilopoulos *et al.*, 2005).

IFN-β and IFN-α expression relies on the activation of nuclear transcription factors NF-kB and/or IRFs. (Merika *et al.*, 2001; Lohoff *et al.*, 2005), and as described in the previous paragraphs, stimulation of some TLR and a few cytosolic PRR activate phosphorylation pathways that induce expression of IFN-α/β. Expression is indeed selective, since TLR2 for instance, whether as a TLR2/TLR1 or a TLR2/TLR6 heterodimer, was found to be unable to induce the expression of IFN-α/β (Kawai *et al.*, 2001; Toshchakov *et al.*, 2002), and Nod2 was not able to stimulate the IRF3-dependent expression of these cytokines in response to intracellular bacteria infection (Stockinger *et al.*, 2004). In contrast, TLR3, TLR4, TLR7/8, TLR9, PKR, RIG-1 and mda-5 can induce IFN-α/β expression. Hence, synthetic dsRNA poly(I:C) was shown to induce the secretion of IFN-α/β through the activation of TLR3 and adaptor TRIF (Hoebe *et al.*, 2003b), or, when located in the cytoplasm, through the activation of PKR (Diebold *et al.*, 2003). Secretion of IFN-α/β was also demonstrated in response to stimulation of RIG-1 by infection with Newcastle disease virus (NDV), which is an RNA virus (Kato *et al.*, 2005), and in response to stimulation of mda-5 by poly(I:C) (Andrejeva *et al.*, 2004). LPS from enterobacteria, lipid A, and vesicular stomatitis virus (VSV), were shown to induce secretion of IFN-α/β through CD14-dependent activation of TLR4 (Jiang *et al.*, 2005), and through adaptor TRIF (Hoebe *et al.*, 2003b). Secretion of IFN-α/β in response to imidazoquinoline R-848 (Hemmi *et al.*, 2002) and ssRNA (Diebold *et al.*, 2004) is

mediated by the activation of TLR7. Infection with influenza virus (Diebold *et al.*, 2004) or NDV (Kato *et al.*, 2005) could also induce IFN-α/β secretion through TLR7 and adaptor MyD88. It was shown that CpG DNA induce secretion of IFN-α/β through TLR9 and adaptor MyD88 (Honda *et al.*, 2005a).

The innate immune system hence relies on different combinations of receptors to stimulate signalling receptors that in turn use different combinations of TIR domain adaptors to activate the transcription of specific sets of genes, coding for innate immune effector functions. Besides the activation of inflammation and microbicidal pathways (Schaefer *et al.*, 2004; Remer *et al.*, 2005), PAMP recognition can induce the expression of immunomodulatory cytokines, such as IFN-α/β, and costimulatory molecules that influence the priming of adaptive immunity (Dalpke *et al.*, 2002; Iwasaki *et al.*, 2004). Dendritic cells in particular are innate cells that mediate the translation of pathogen recognition by the innate immune system into adaptive immunity (Reis e Sousa, 2004).

1.2 Activation of adaptive immunity by dendritic cells

Dendritic cells (DC) are professional antigen presenting cells (APC) that play an important role in regulating adaptive immune responses, by tolerising or activating naïve T cells (Steinman *et al.*, 1980; Banchereau *et al.*, 1998; Banchereau *et al.*, 2000; Steinman *et al.*, 2003). DC inspect their environment by ingesting surrounding materials, and processing them into peptides that are presented to T cells. Concomitantly, DC integrate signals that may be emitted as a result of the recognition of PAMPs or other signs of danger (Matzinger, 1994), and accordingly, either prime T cells or render them non-functional. Processes of antigen presentation and priming are described below.

1.2.1 Antigen presentation

1.2.1.1 Antigen uptake

DC use various mechanisms to internalise antigens (Underhill *et al.*, 2002). Large volumes of soluble antigens are efficiently internalised by macropinocytosis (Sallusto *et al.*, 1995), which is an actin-dependent process. Phagocytosis is also a cytoskeleton-dependent type of internalisation, but is initiated by the engagement of

specific receptors, which induce actin polymerisation and antigen uptake (Greenberg *et al.*, 2002). Endocytic receptors include some C-type lectins, such as the mannose receptor and DEC-205 (Jiang *et al.*, 1995; Sallusto *et al.*, 1995), FcγR (Lanzavecchia, 1990; Regnault *et al.*, 1999), CR3 (Underhill *et al.*, 2002; Gasque, 2004) and scavenger receptor CD36 (Platt *et al.*, 1998; Gough *et al.*, 2000; Stuart *et al.*, 2005). Actin assembly during phagocytosis is induced through a family of guanosine triphosphatases (GTPases) (Etienne-Manneville *et al.*, 2002; Greenberg *et al.*, 2002), and a role for TLR stimuli in enhancement of actin assembly has been reported (West *et al.*, 2004). Endocytic receptors can also mediate actin-independent uptake of macromolecules through clathrin-coated vesicles. Internalised antigens are then processed through the MHC II presentation pathways.

1.2.1.2 Antigen processing in the MHC II pathway

DC state of activation regulates MHC II-restricted antigen presentation. Indeed, immature DC retain antigens in late lysosomes, where a suboptimal pH slows the proteolytic degradation of antigens and of MHC II invariant chain Ii, which blocks MHC II binding groove (Lennon-Dumenil *et al.*, 2002). In contrast, DC maturation (Banchereau *et al.*, 2000; Mazzoni *et al.*, 2004) induces a better acidified environment, and digested peptides and possibly large antigen fragments (Trombetta *et al.*, 2005) bind rapidly to MHC II. The stable peptide-MHC II complexes are then transported to endosomal vesicles where they colocalise with costimulatory molecules, before being targeted to the cell membrane where they present antigenic peptides to $CD4^+$ T cells (Guermonprez *et al.*, 2002). PAMPs and inflammatory signals such as cytokines, which induce DC maturation, also regulate the formation of peptide-MHC II complexes (Inaba *et al.*, 2000; Manickasingham *et al.*, 2000; Turley *et al.*, 2000; Fiebiger *et al.*, 2001; Thery *et al.*, 2001).

Antigen presentation to $CD8^+$ T cells occurs via the MHC I pathway, as described below.

1.2.1.3 Antigen-processing in the MHC I pathways

Endogenous cytosolic proteins are degraded into peptides by a large catalytic multisubunit protease complex called the proteasome (Dick *et al.*, 1994; Guermonprez *et al.*, 2002). Peptides are transferred to the endoplasmic reticulum (ER) by specialised transporters (transporters associated with antigen presentation,

TAP). In the lumen of the ER, a chaperone-mediated assembly generates a stable complex containing MHC I, β2-microglobulin and the peptide (Li *et al.*, 2002). This complex then leaves the ER and is transported through the Golgi apparatus to the cell surface, where it can present the peptide to $CD8^+$ T cells.

It had been observed that $CD8^+$ T cells could be primed against exogenously acquired antigens (Bevan, 1976a; Bevan, 1976b). It was then shown that peptides generated from endocytosed antigens could gain access to the cytosol and be processed through the MHC I presentation pathway, a process termed cross-presentation (Pfeifer *et al.*, 1993; Kovacsovics-Bankowski *et al.*, 1995; Reis e Sousa *et al.*, 1995; Kurts *et al.*, 1996; Heath *et al.*, 2001). Three internalisation pathways may lead to cross-presentation. Macropinocytosis allows constitutive receptor-independent cross-presentation of soluble antigens (Norbury *et al.*, 1995; Brossart *et al.*, 1997; Norbury *et al.*, 1997). Recently, peptides transferred from one cell to another through gap junctions were found to be cross-presented (Neijssen *et al.*, 2005). However, the major route for antigen uptake and cross-presentation may be phagocytosis (Kovacsovics-Bankowski *et al.*, 1995; Gagnon *et al.*, 2002). Phagocytosis of bacteria (Weiskirch *et al.*, 1997; Rescigno *et al.*, 1998), of apoptotic and necrotic cells (Albert *et al.*, 1998; Fonteneau *et al.*, 2002), and antibody-opsonised antigens (Regnault *et al.*, 1999; Rodriguez *et al.*, 1999) can provide antigens that are cross-presented. Particulate and cell-associated antigens have actually been found to be cross-presented more efficiently than soluble antigens (Kovacsovics-Bankowski *et al.*, 1993; Li *et al.*, 2001). Cell-derived fragments, such as carried by exosomes (Thery *et al.*, 2002), may be a source of antigens that can be cross-presented (Wolfers *et al.*, 2001). Heat shock protein (Hsp), released from necrotic cells for instance, have also been shown to convey antigens and mediate cross-presentation (Singh-Jasuja *et al.*, 2000; Binder *et al.*, 2001; Binder *et al.*, 2005).

Two pathways for peptide loading onto MHC I, TAP and proteasome-dependent or -independent, have been described (Castellino *et al.*, 2000; Sigal *et al.*, 2000; Norbury *et al.*, 2001; Ackerman *et al.*, 2004). The TAP-proteasome-dependent pathway is considered dominant, since more efficient. Both phagocytosed and pinocytosed antigens can get into contact with ER-like compartments, where they are processed for MHC I loading (Gagnon *et al.*, 2002; Ackerman *et al.*, 2005). Indeed, the ER can

donate some of its membrane to phagosomes, which become equipped for self-sufficient cross-presentation (Ackerman *et al.*, 2003; Guermonprez *et al.*, 2003; Houde *et al.*, 2003). Soluble antigens in pinosomes are delivered to the ER, possibly by active retrieval or by membrane fusion (Ackerman *et al.*, 2005).

While macrophages (Kovacsovics-Bankowski *et al.*, 1993; Reis e Sousa *et al.*, 1995; Castellino *et al.*, 2000), B cells (Ke *et al.*, 1996; Hon *et al.*, 2005) and DC (Rock *et al.*, 1993; Regnault *et al.*, 1999) were all found to cross-present antigens, it was demonstrated that DC were sufficient and necessary for cross-presentation and functional activation of $CD8^+$ T cells to take place (Kurts *et al.*, 2001; Jung *et al.*, 2002). DC are constitutively able to cross-present, but DC maturation, induced by innate signals, may enhance this process (Datta *et al.*, 2003; Gil-Torregrosa *et al.*, 2004). $CD11c^+$ DC have been divided into two subsets according to the expression of CD8α and myeloid marker CD11b (Pulendran *et al.*, 1997; Vremec *et al.*, 2000). $CD11b^{high}CD8\alpha^-CD4^+$ and $CD11b^{high}CD8\alpha^-CD4^-$ DC have been classed as "myeloid" DC, while $CD11b^{low}CD8\alpha^+$ have been referred to as "lymphoid" DC. "Lymphoid" DC were first described as the only subset able to cross-present antigens (den Haan *et al.*, 2000; Pooley *et al.*, 2001), probably through its capacity to endocytose dead cells (Iyoda *et al.*, 2002; Schulz *et al.*, 2002). Actin-dependent mechanisms are involved in uptake of apoptotic cells, and selective inhibition of Rac1 GTPase expression in DC was found to result in decreased uptake of dying cells by $CD8\alpha^+$ DC, and decreased cross-presentation of cell-associated exogenous antigen (Kerksiek *et al.*, 2005). "Myeloid" DC were also shown to be able to mediate cross-presentation in the mesenteric lymph node (Chung *et al.*, 2005), and in the spleen provided that the cells were activated through the FcγR by antibody-opsonised antigens (den Haan *et al.*, 2002). In contrast, $CD8\alpha^-CD4^+$ myeloid DC may be more efficient at presenting antigens to $CD4^+$ T cells (Pooley *et al.*, 2001; den Haan *et al.*, 2002).

Antigen presentation to $CD4^+$ T cells or $CD8^+$ T cells does not necessarily result in functional T cell responses. Requirements for effective priming and cross-priming are described below.

1.2.2 Antigen-specific priming of T cells and B cells

T cell receptor (TCR) and B cell receptor (BCR) possess a highly variable antigen-binding site, to provide a repertoire that can ensure recognition of some antigens from possibly nearly every pathogen. Binding of a specific antigen by the TCR or BCR however is not sufficient to induce T cell or B cell clonal expansion. Indeed, both cell type need to receive a costimulatory signal, also referred to as second signal.

1.2.2.1 $CD4^+$ T cell priming

TCR on $CD4^+$ T cells recognise peptides, processed from antigens and presented by APC, in the context of MHC II. The second signal required by T cells for proliferation is the stimulation of costimulatory molecule CD28 by members of the B7 family (CD80 and CD86 molecules) present on mature APC (Lenschow *et al.*, 1996; Janeway *et al.*, 1998). Stimulation of CD28 activates IL-2 and IL-2 receptor synthesis, which signal the T cells to proliferate (Jenkins *et al.*, 1991; Cerdan *et al.*, 1992; Cerdan *et al.*, 1995). DC maturation is stimulated by signals induced by the recognition of pathogens for instance, and many PAMPs, including peptidoglycan, LTA, poly(I:C), LPS from *E. coli*, flagellin and CpG DNA have been shown to induce upregulation of CD80 and CD86 expression on DC *in vivo* and *in vitro* (Datta *et al.*, 2003; Schwarz *et al.*, 2003). It was demonstrated for example that flagellin from *Salmonella* was able to enhance antigen-specific $CD4^+$ T cell expansion, through the induction of CD80 and CD86 expression (McSorley *et al.*, 2002). Other PAMPs, such as LPS, have also been shown to augment antigen-specific $CD4^+$ T cell proliferation and differentiation, in a model of adoptive transfer of $CD4^+$ TCR transgenic T cells specific for a protein antigen (Pape *et al.*, 1997; Pulendran *et al.*, 2001).

In addition to providing signal 1 (antigen-MHC II complex) and signal 2 (costimulatory interactions) for $CD4^+$ T cell priming, DC are also central to $CD4^+$ T cell differentiation into different types of effector cells, by providing what is sometimes referred to as signal 3: cytokines that polarise $CD4^+$ T cell differentiation into type 1 (Th1) or type 2 (Th2) T helpers cells; the function of Th1 and Th2 cells will be described later on (Mosmann *et al.*, 1989; Kalinski *et al.*, 1999). In response to microbial stimuli, including LPS from *E. coli*, DC are able to rapidly produce IL-

12 (Macatonia *et al.*, 1995; Trinchieri, 1995; Heufler *et al.*, 1996; Magram *et al.*, 1996; Reis e Sousa *et al.*, 1997; Morelli *et al.*, 2001), which is a major factor in the development of Th1 cells (Macatonia *et al.*, 1995; Trinchieri, 1995; Magram *et al.*, 1996). Early IL-12 production allows DC to induce, in synergy with IL-18 (Okamura *et al.*, 1995), the secretion of IFN-γ by innate cells (Ohteki *et al.*, 1999; Fukao *et al.*, 2000; Stober *et al.*, 2001; Kamath *et al.*, 2005), which facilitates Th1 differentiation (Stoll *et al.*, 1998; Yoshimoto *et al.*, 1998). IFN-γ hence contributes to the amplification process of priming DC into IL-12 producing cells (Vieira *et al.*, 2000). In the presence of microbial stimuli such as heat-killed bacteria, parasite antigen or CpG DNA, IL-12-primed Th1 reciprocally signal DC to terminally mature into Th1-priming cells, by triggering IL-12 production through CD40 signalling (Snijders *et al.*, 1998; Schulz *et al.*, 2000; Quezada *et al.*, 2004). Although IFN-α/β alone are not sufficient to directly induce antigen-specific Th1 development in mice, as opposed to humans (Parronchi *et al.*, 1992; Wenner *et al.*, 1996; Rogge *et al.*, 1997; Kadowaki *et al.*, 2000), it was found that poly(I:C)-activated murine DC can promote Th1 polarisation (Cella *et al.*, 1999; Cella *et al.*, 2000). IFN-α/β were shown to cooperate with IL-18 to prime Th1 cells in response to *S. typhimurium* (Freudenberg *et al.*, 2002), and were shown to enhance the priming of IFN-γ-producing $CD4^+$ T cells (Le Bon *et al.*, 2001). It was also demonstrated that IFN-α/β signalling could regulate IL-12 production from DC in response to viral infection or stimulation with PAMPs (Dalod *et al.*, 2002; Gautier *et al.*, 2005), thereby controlling Th1 polarisation.

IL-4 is a major Th2 cytokine, but was not found to play an important role in DC-mediated differentiation of $CD4^+$ T cells into Th2 cells (Schmitz *et al.*, 1994). In contrast to the cytokine-induced Th1 polarisation, it is possible that Th2 polarisation develops by default, as a result of microbial stimuli that do not stimulate the production of IL-12, or of factors that downregulate production of IL-12, such as IL-10 (Mosmann *et al.*, 1991). In this way, IL-10 is involved in Th2 polarisation in response to *Schistosoma mansoni* for example (Sher *et al.*, 1991; Hoffmann *et al.*, 2000; Patton *et al.*, 2001). Nematode parasite *Nippostrongylus brasiliensis* was shown to drive the differentiation of adoptively transferred $CD4^+$ TCR transgenic T cells into Th2 cells through stimulation of autocrine IL-4 production from antigen-specific Th2 T cells (Liu *et al.*, 2005).

DC are able to induce differential Th polarisation depending on the nature of the microbial stimulus. Indeed, TLR enable DC to distinguish between PAMPs. Therefore, while LPS from *P. gingivalis*, which is recognised by TLR2 (Hirschfeld *et al.*, 2001), was not able to induce IL-12 production from DC and induced the differentiation of antigen-specific T cells into Th2 cells, LPS from *E. coli* stimulated a TLR4-dependent production of IL-12 from DC and induced Th1 polarisation of antigen-specific T cells (Pulendran *et al.*, 2001). DC activated by TLR2 agonist lipopeptide Pam_3CSK_4 (Takeuchi *et al.*, 2002) similarly induced an antigen-specific Th2 polarisation of $CD4^+$ TCR transgenic T cells (Dillon *et al.*, 2004).

One of the main functions of primed $CD4^+$ T cells is the activation of antigen-specific B cells.

1.2.2.2 B cell priming

The BCR recognises motifs on pathogens. Upon BCR binding by a protein antigen, B cells behave as APC. Indeed, the antigen-BCR complex is internalised and the BCR delivers the antigen to endosomes where it is processed into peptides that are then loaded onto MHC II molecules (Lanzavecchia, 1985; Mitchell *et al.*, 1995; Stoddart *et al.*, 2005; Trombetta *et al.*, 2005). This mechanism allows B cells to present peptides from protein antigens to $CD4^+$ T cells and receive accessory signals from DC-primed $CD4^+$ T cells that are specific for the same antigen. Accessory signals are indeed required for B cell priming against protein antigens (Parker, 1993). Signalling through CD40 on B cells in particular is crucial for B cell activation in response to protein antigens (Lane *et al.*, 1993; Nonoyama *et al.*, 1993; van Essen *et al.*, 1995; Lee *et al.*, 2003b). CD40 on B cells is required for immunoglobulin isotype switching, as illustrated by the X-linked hyper IgM syndrome (Aruffo *et al.*, 1993; Durandy *et al.*, 1993; Kawabe *et al.*, 1994). The use of anti-CD40 antibodies has proved that CD40 stimulation supports antigen-specific B cell-responses by both inducing $CD4^+$ T cell help and directly activating B cells (Valle *et al.*, 1989; Carlring *et al.*, 2004; Barr *et al.*, 2005).

It has been demonstrated that IFN-α/β enhance B cell responses and promote isotype switching (Le Bon *et al.*, 2001). The same study also showed that DC stimulated with IFN-α/β were sufficient to mediate these effects.

It has been reported that B cells can be primed directly by DC, which possibly provide cell-contact and cytokine-mediated signals (Dubois *et al.*, 1997; Wykes *et al.*, 1998).

Some microbial carbohydrate structures, including polysaccharides and nucleic acids, can activate and costimulate B cells directly, by cross-linking the BCR through multivalent motifs (Dintzis *et al.*, 1982), or by simultaneously binding the BCR and TLR expressed by B cells. Polysaccharide antigens can activate the complement cascade through various pathways that generate cleavage products of complement protein C3 (Griffioen *et al.*, 1991; Carroll, 1998). C3b and C3d for instance opsonise microbial carbohydrates and can activate B cells through the BCR and co-receptor complex, composed of CD19 and complement receptor 1 (CR1, also known as CD35), which binds C3b, and CR2 (CD21), which binds C3d (Fearon *et al.*, 2000; Haas *et al.*, 2002; Pozdnyakova *et al.*, 2003). CR2 was shown to also play an important role in B cell activation against protein antigens (Ahearn *et al.*, 1996; Molina *et al.*, 1996). C3b and C3d were found to enhance B cell priming against protein antigens (Ross *et al.*, 2000; Villiers *et al.*, 2003; Bower *et al.*, 2004; Haas *et al.*, 2004).

B cells can be activated by TLR agonists: by lipopetide MALP-2 through TLR2 (Borsutzky *et al.*, 2005), by CpG DNA through TLR9 (Bernasconi *et al.*, 2003) and by LPS through TLR-related receptor complex RP105/MD-1 (Ogata *et al.*, 2000; Nagai *et al.*, 2002; Yazawa *et al.*, 2003). A synergistic effect of signalling through TLR9 and CD40 on B cell activation has been reported (Gantner *et al.*, 2003b). Stimulation of TLR2, TLR9 or RP105 can also lead to B cell priming against protein antigens (Borsutzky *et al.*, 2005; Nagai *et al.*, 2005; Tudor *et al.*, 2005).

1.2.2.3 $CD8^+$ T cell priming

1.2.2.3.1 Direct priming

$CD8^+$ T cell TCR recognises peptides, processed from antigens and presented by APC, in the context of MHC I. As for $CD4^+$ T cells, stimulation of costimulatory molecule CD28 by CD80 and CD86 on mature APC provides the second signal required by T cells for proliferation. However, in addition to TCR stimulation by peptide-MCH I complexes and CD28 activation by B7 molecules, $CD8^+$ T cell priming may require other signals, depending on the nature of the pathogen. Indeed,

$CD8^+$ T cells may or may not also require $CD4^+$ T cell help (Ahmed *et al.*, 1988). Priming of $CD8^+$ T cells against some viruses, such as γ-herpesvirus (Cardin *et al.*, 1996) and Herpes simplex virus 1 (HSV-1) (Jennings *et al.*, 1991) is dependent on $CD4^+$ help, while priming against mousepox virus (Buller *et al.*, 1987), influenza virus (Liu *et al.*, 1989), Sendai virus (Hou *et al.*, 1992), lymphocytic choriomeningitis virus (LCMV) (Borrow *et al.*, 1996; Whitmire *et al.*, 1999) or VSV (Andreasen *et al.*, 2000; McAdam *et al.*, 2000), and against some intracellular bacteria such as *Listeria monocytogenes* (Shedlock *et al.*, 2003b), is independent of $CD4^+$ T cell help. CD4-independent $CD8^+$ T cell priming probably reflects the properties of some pathogens to stimulate strong DC activation and survival, through PAMP-induced signals for example (Schuurhuis *et al.*, 2000), and conditioned DC would then be able to induce $CD8^+$ T cell priming, through costimulatory signals from CD40/CD40 ligand (CD40L, also known as CD154) (Lee *et al.*, 2003a) or B7/CD28 (Andreasen *et al.*, 2000; McAdam *et al.*, 2000) interactions, and cytokine production (Adam *et al.*, 2005) for instance.

In some cases of chronic viral infection, $CD4^+$ T cell help may consist in IL-2 production that prolongs $CD8^+$ T cell activation (Matloubian *et al.*, 1994). In most cases, $CD4^+$ T cells provide help by activating the APC that presents the antigen to the $CD8^+$ T cell, and the conditioned DC then stimulates the $CD8^+$ T cell, a process termed licensing (Guerder *et al.*, 1992; Lanzavecchia, 1998). It has been shown that $CD8^+$ T cell responses against HSV-1 for example depend on cognate licensing of DC by $CD4^+$ T cells (Smith *et al.*, 2004). It has been demonstrated that interactions between CD40L on $CD4^+$ T cells and CD40 on DC can activate DC to license $CD8^+$ T cell to develop effector functions (Ridge *et al.*, 1998). Therefore $CD4^+$ T cell-dependent $CD8^+$ T cell priming against viruses such as HSV-1 may also occur through CD40L/CD40-mediated activation of DC. CD40, which expression on DC is upregulated by PAMPs (Schwarz *et al.*, 2003), is a costimulatory molecule that belongs to the TNF receptor superfamily (Quezada *et al.*, 2004). $CD4^+$ T cell-mediated signalling through CD40 induces further DC activation. Activation of DC can trigger the expression of other costimulatory molecules from the TNF receptor family, such as CD70 (Watts, 2005). CD70 interaction with its ligand CD27 on $CD8^+$ T cells has been shown to contribute to the activation of $CD8^+$ T cell effector functions (Hendriks *et al.*, 2000; Hendriks *et al.*, 2003; Borst *et al.*, 2005).

In addition to the three-cell ($CD4^+$ T cell-DC-$CD8^+$ T cell) model of $CD4^+$ T cell help for $CD8^+$ T cell priming, it has been shown that $CD4^+$ T cells can activate $CD8^+$ T cells directly. It has been demonstrated that T cells, through their TCR complex, absorb molecules from APC, including peptide-MHC complexes (Huang *et al.*, 1999; Hwang *et al.*, 2000) and costimulatory molecules (Xiang *et al.*, 2005). $CD4^+$ T cells in particular that acquire peptide-MHC I complexes have been shown to be able to prime $CD8^+$ T cells (Kennedy *et al.*, 2005; Xiang *et al.*, 2005). $CD4^+$ T cells may also signal directly to $CD8^+$ T cells, which can express CD40, through CD40L/CD40 interactions (Bourgeois *et al.*, 2002a).

When specific conditions are met, $CD8^+$ T can be primed against antigens that are cross-presented, through a process termed cross-priming (Yewdell *et al.*, 2005).

1.2.2.3.2 Cross-priming

As described in paragraph 1.2.1.3, antigens captured by APC can be processed through the MHC I presentation pathway. However, the generation of functional $CD8^+$ T cells responses through cross-priming is controlled. Indeed, it was found, using the rat insulin promoter (RIP)-mOVA transgenic mouse model (Kurts *et al.*, 1996), that cross-presentation of self-antigen led to tolerance mechanisms rather than cross-priming of OVA-specific $CD8^+$ T cells (Kurts *et al.*, 1997). OVA-specific $CD8^+$ T cell tolerance was also observed when OVA, exogenously administered and targeted to DC through the endocytic receptor DEC-205, was cross-presented (Bonifaz *et al.*, 2002). In a model of mouse tumour cells expressing the defined adenovirus ad5E1A antigen, no $CD8^+$ T cell responses were elicited *in vivo* against the tumour, whilst E1A was cross-presented (van Mierlo *et al.*, 2004). In contrast, administration of OVA in combination with microbial products, such as CFA (Bennett *et al.*, 1997) or CpG DNA (Cho *et al.*, 2000), injection of monophosphoryl lipid A (MPL) or CpG DNA into ad5E1A-expressing-tumour bearing mice (van Mierlo *et al.*, 2004), or induction of tumour cell apoptosis *in vivo* (Nowak *et al.*, 2003), induced cross-priming against the soluble or tumour cell-associated antigens. dsRNA, present in virally infected cells, also promotes cross-priming against virus-expressed antigens (Schulz *et al.*, 2005).

Since $CD8^+$ T cell cross-priming is not constitutive, it has been hypothesised that, as for $CD8^+$ T cell direct priming during presentation of endogenous antigens, licensing

mechanisms control the onset of $CD8^+$ T cell effector activities against cross-presented antigens (Lanzavecchia, 1998). It was found that in the case of cross-presentation of cell-associated antigens, such as OVA-loaded splenocytes (Bennett *et al.*, 1997), Ad5E1-expressing allogeneic cells (Schoenberger *et al.*, 1998) and male antigen H-Y-expressing cells (Ridge *et al.*, 1998), cross-priming required cognate $CD4^+$ T cell help. It was demonstrated in CD40- and CD40L-deficient mice that $CD4^+$ T cell help was mediated through CD40L/CD40 interactions (Bennett *et al.*, 1998). The use of activating anti-CD40, or/and CD40L-blocking antibodies (Schoenberger *et al.*, 1998), in $CD4^+$ T cell-depleted mice (Snapper *et al.*, 1987; Bennett *et al.*, 1998) showed that $CD4^+$ T cells stimulated CD40 signalling to induce cross-priming against cell-associated antigens (cellular cross-priming).

DC, activated *in vitro* by anti-CD40 antibodies, were found to be able to induce cellular cross-priming (Ridge *et al.*, 1998), while B cells were shown to be dispensable *in vivo* (Schoenberger *et al.*, 1998). Selective diphteria toxin-induced apoptosis of CD11c cells *in vivo* demonstrated that DC mediated the licensing of adoptively transferred $CD8^+$ TCR transgenic T cells against Malaria sporozoite-infected cells (Jung *et al.*, 2002). In a tumour model, adoptive transfer of CD40-proficient and CD40-deficient adE1A-specific TCR transgenic $CD8^+$ T cells into tumour-bearing CD40-deficient or wild-type mice showed that CD40 expression on $CD8^+$ T cells was not required for cross-priming (van Mierlo *et al.*, 2004). Those experiments support the hypothesis that cellular cross-priming can be licensed by the conditioning of DC by antigen-specific $CD4^+$ T cells through CD40 signalling. How stimulation of CD40 on DC conditions them to license cross-priming is unclear, although it has been suggested that CD40L-mediated activation of DC favours induction of cross-priming through other costimulatory molecules interactions, such B7/CD28 and CD70/CD27, since antibody-blocking of B7 molecules and CD70 on DC impaired cross-priming (Bullock *et al.*, 2005).

Although CD80 and CD86 may not be sufficient individually to generate signals that license cellular cross-priming, these costimulatory molecules were found to be involved in the licensing process when both were stimulated (Ridge *et al.*, 1998). Using B7-and CD28-deficient mice, it has been demonstrated that signalling through CD28, even in the absence of $CD4^+$ T cells, contributes to licensing of cellular cross-priming (Prilliman *et al.*, 2002).

$CD8^+$ T cells can be cross-primed against soluble antigens in the presence of some microbial stimuli, in which case licensing was found to be $CD4^+$ T cell-independent. Hence, CFA and CpG DNA induced cross-priming against soluble antigens in $CD4^+$ T cell-depleted mice or CD4-deficient mice (Bennett *et al.*, 1997; Sparwasser *et al.*, 2000). It is thought that these stimuli bypass requirements for $CD4^+$ T cell help by conditioning DC in their own way for licensing of cross-priming. CpG DNA was shown to be able to induce cross-priming independently of CD40 or CD40L, while costimulation with B7 molecules was required (Cho *et al.*, 2002).

It has been suggested that CD40-independent pathways of DC activation for cross-priming involve soluble factors such as cytokines (Wild *et al.*, 1999; Lu *et al.*, 2000). IL-12 for instance was shown to contribute to the induction of cross-priming against a soluble antigen by CpG DNA (Cho *et al.*, 2002). The role of IFN-α/β was also investigated, and induction of cross-priming by CpG DNA was shown to partially depend on IFN-α/β receptor signalling (Van Uden *et al.*, 2001; Cho *et al.*, 2002). IFN-α/β was characterised as an important soluble licensing stimulus, as viral infection-induced IFN-α/β was shown to induce cross-priming against a soluble antigen, and DC activation by IFN-α was shown to license cross-priming (Le Bon *et al.*, 2003). It was also demonstrated that IFN-α was able to license cross-priming in the absence of $CD4^+$ T cells and independently of CD40-mediated activation. Furthermore, IFN-α was able to provide signals that could replace the $CD4^+$ T cell help required for induction of cellular cross-priming, since cross-priming against a cell-associated antigen could be licensed, in the presence of IFN-α, in MHC $II^{-/-}$-deficient mice.

Whether or not initial priming of $CD8^+$ T cell effector functions can occur in the absence of $CD4^+$ T cells, it is now known that $CD4^+$ T cells are crucial for the development of $CD8^+$ T cell memory, which contributes to protective immunity against reinfection (Bourgeois *et al.*, 2002a; Bourgeois *et al.*, 2002b; Janssen *et al.*, 2003; Shedlock *et al.*, 2003a; Sun *et al.*, 2003). $CD4^+$ T cells therefore play a major role in orchestrating the adaptive immune response, which is crucial to the defence against pathogens, as illustrated by the severe combined immunodeficiency (SCID) phenotype, where absence/impairment of T and B cell functions results in susceptibility to all classes of pathogens (Mead *et al.*, 1991; Chang *et al.*, 1994; Rottenberg *et al.*, 1999; Ellison *et al.*, 2000; Bitsaktsis *et al.*, 2004).

1.3 The adaptive immune response

T and B cells, once activated in the appropriate conditions, are primed, and thereby differentiate into cells that perform specific effector functions, which cooperate to eliminate pathogens.

1.3.1 T helper cell responses

$CD4^+$ T helper cells play a strategic role. Their function is to activate and control the defence mechanisms that are appropriate for killing the invading pathogen. $CD4^+$ T cell priming by DC determines whether T cells differentiate into Th1 or Th2 cells. Th1 and Th2 subsets differ in the cytokines they produce, and thereby activate distinct types of adaptive responses. Th1 cells are characterised primarily by the secretion of IL-2, IFN-γ and TNF-β (Mosmann *et al.*, 1986; Constant *et al.*, 1997; Murphy *et al.*, 2002). IL-2 acts on Th1 cells themselves to sustain the clonal expansion and therefore increase the number of effector cells (Bodeker *et al.*, 1980; Minami *et al.*, 1993). Th1 cells coordinate cytotoxic mechanisms that are directed against phagocytosed pathogens. Macrophages are phagocytes that can also process and present antigens in the context of MHC II. Th1 cells that recognise peptide-MHC II complexes enhance macrophage functions by signalling through CD40L/CD40 interactions and by secreting cytokines, such as IFN-γ and TNF-β, at close range (Nathan *et al.*, 1983; Seid *et al.*, 1986; Orme *et al.*, 1993; Soong *et al.*, 1996; Mencacci *et al.*, 1998; Yamauchi *et al.*, 2000). Activated macrophages increase their antigen processing activities, which results in lytic enzyme-mediated killing of pathogens and contributes to the priming of more $CD4^+$ T cells. Macrophages also secrete IL-12, which promotes Th1 polarisation, and increase production of microbicidal metabolites, such as nitric oxide (NO) and oxygen free radicals (Munoz-Fernandez *et al.*, 1992; Ehlers *et al.*, 2003; Shrivastava *et al.*, 2004). If macrophages fail to digest phagocytosed pathogens, Th1 cells can induce apoptosis of the infected macrophage by binding Fas through its Fas ligand, which triggers signalling pathways that lead to DNA degradation and cell death (Hahn *et al.*, 1995). IFN-γ secretion by Th1 cells also activates NK cells, which contribute to the killing of pathogen-infected cells by inducing apoptosis, through a process called antibody-dependent cell-mediated cytotoxicity (ADCC). Particular antibody isotypes mediate

ADCC, and Th1 cells can promote the production of appropriate antibodies by inducing B cell antibody isotype switching (Stevens *et al.*, 1988), through CD40L/CD40 interactions and the secretion of IFN-γ (Gracie *et al.*, 1996; Sulica *et al.*, 2001).

Th2 cells in contrast are characterised primarily by the secretion of IL-4, IL-5, IL-9 and IL-13 (Seder *et al.*, 1994). IL-4, in addition to driving the clonal expansion of Th2 cells (Fernandez-Botran *et al.*, 1986; Kim *et al.*, 2005b), activates B cells in the presence of CD40 signalling, which results in increased B cell antigen presentation capacity, proliferation and differentiation into antibody-secreting plasma cells. IL-5 and IL-13 also contribute in stimulating B cell growth and differentiation. IL-4 also promotes antibody isotype switching to IgG1 and IgE (Snapper *et al.*, 1987; Lebman *et al.*, 1988), which participate in neutralising pathogen, to prevent infection, and in killing pathogens that have not infect cells. IgE-mediated responses are supported by IL-5, IL-9 and IL-13, as IL-5 induces differentiation of eosinophils (Yokota *et al.*, 1987) and IL-9 enhances mast cells activity (Hultner *et al.*, 1990) while potentiating the effects of IL-4 (Petit-Frere *et al.*, 1993). IL-13 and IL-10 contribute to the inhibition of Th1 responses by downregulating macrophage inflammatory responses (Bogdan *et al.*, 1993; de Waal Malefyt *et al.*, 1993).

Th1 and Th2 $CD4^+$ T cells therefore orchestrate the adaptive immune response, by directing the activation of cells which functions are most appropriate for the elimination of pathogens. Th1 cells primarily support cell-mediated immune responses that can kill pathogens that are within cells, while Th2 cells induce humoural responses that mediate killing of extracellular pathogens.
Antibodies, produced by B cells, contribute to many Th1- and Th2-induced pathogen killing mechanisms.

1.3.2 Antibody responses

Antibodies are secreted by B cells that have recognised the antigen they are specific for, and have received the appropriate signals to differentiate into plasmablasts, and later into plasma cells. In the early stages of the response, mostly IgM antibodies, which initially serve as BCR on naïve B cells, are secreted. In response to various differentiation signals, including cytokines and CD40/CD40L interactions, plasmablasts undergo isotype switching and express immunoglobulins of a different

isotype. Isotypes have specialised functions. IgM can form pentamers (Parkhouse *et al.*, 1970; Brewer *et al.*, 1997), and are thereby suited to recognise repeating antigens, such as presented by microbial polysaccharides. The pentameric form also efficiently activates the complement classical pathway (Davis *et al.*, 1989; Taylor *et al.*, 1994). Indeed, the Fc portions of pentamers bound to microbial surfaces are in the most appropriate spatial arrangement to bind complement protein C1q and induce the conformational change that will activate the complement cascade, resulting in pathogen killing by the membrane-attack complex. IgG2a antibodies in mice have also a particular ability to activate complement-mediated killing (Oishi *et al.*, 1992; Sato *et al.*, 1997). IgG2a antibodies bound to pathogens are also able to induce killing by cross-linking Fc receptors on macrophages and NK cells (Kipps *et al.*, 1985). Cross-linking of FcγRIII, as well as FcγRI on macrophages, activates intracellular signalling pathways leading to the release of inflammatory and toxic molecules (Sanchez-Mejorada *et al.*, 1998). Macrophages express NO, oxygen free radicals, antimicrobial peptides and proteases, and NK cells, which like cytotoxic T cells contain lytic granules, secrete antimicrobial and apoptosis-inducing molecules (Bonnema *et al.*, 1994; Jensen *et al.*, 1998; Stenger *et al.*, 1998; Ernst *et al.*, 2000; Ochoa *et al.*, 2001), resulting in the killing of antibody-coated target pathogens or cells. FcγRI can also be activated on phagocytes by IgG1 antibodies. IgG1 efficiently opsonise pathogens by coating them and targeting them to phagocytes through their Fc portion. Signalling through FcγRI induces phagocytosis and killing of pathogens by enzymes in lysosomes. The killing of extracellular pathogens, parasitic helminth in particular, can be mediated by IgE antibodies, which coat parasites and activate eosinophils. Indeed, cross-linking of FcεR on eosinophils (Kinet, 1999), among other mechanisms (Arase *et al.*, 2003), triggers degranulation that can result in the death of parasites.

While humoral responses mediate mechanisms effective at killing extracellular pathogens, cytotoxic $CD8^+$ T cells are specialised in eliminating intracellular pathogens by instructing the infected cell to commit suicide.

1.3.3 Cytotoxic $CD8^+$ T lymphocyte responses

$CD8^+$ T cells that have been primed by the appropriate signals while recognising their specific antigen differentiate into CTL that are equipped for target cell killing

(Oehen *et al.*, 1998; Opferman *et al.*, 1999; Morishima *et al.*, 2005) and cytokine secretion (Erard *et al.*, 1993; Sad *et al.*, 1995; Kemp *et al.*, 2001; Kemp *et al.*, 2005).

Upon TCR activation, and depending on the cytokine environment, CTL locally and systemically secrete cytokines such as IFN-γ, TNF-α, IL-4, IL-5 and IL-10, which may participate in the immunomodulation of the response (Prezzi *et al.*, 2001; Woodland *et al.*, 2003; Beadling *et al.*, 2005), and, in the case of IFN-γ and TNF-α, contribute to the response against intracellular pathogens (Ramshaw *et al.*, 1997; White *et al.*, 2000; Lichterfeld *et al.*, 2004). These cytokines can induce the expression of cytosolic proteins that decrease transcription and/or replication of several viruses (Wong *et al.*, 1986; Slifka *et al.*, 2000). Cytokines also enhance MHC I expression and antigen exposure, recruitment of cells such as macrophages, and enhancement of macrophage antigen presentation and antimicrobial activities. These cytokine-induced mechanisms contribute to limiting the spread of intracellular pathogens, supporting the primary effector function of CTL, which is to kill infected cells.

CTL store perforin, granzymes and Fas ligand in lytic granules, which are part of the cell's secretory lysosome system (Clark *et al.*, 2003). When effector cells re-encounter the antigen on MHC I of infected cells, TCR activation triggers a polarisation of the lytic granules that concentrate at a focal point by the TCR. The content of the granules is thereby secreted by exocytosis specifically towards the target cell, in the tight space at the point of contact between CTL and target cell. Exocytosis also controls some of the surface exposure of Fas ligand (Bossi *et al.*, 1999; Haddad *et al.*, 2001). Target cells are killed by being instructed to undergo programmed cell death, termed apoptosis. CTL can use two mechanisms to induce apoptosis: by engaging death receptors on target cells, or by releasing the contents of lytic granules. Signalling through death receptor Fas on target cells, activated by Fas ligand on CTL, induces cell death through proteolytic cascades that result in the breakdown of the nuclear membrane and DNA degradation (Simon *et al.*, 2000; Thorburn, 2004). It is thought however that CTL killing mechanisms are principally mediated by granzymes and perforin (Barry *et al.*, 2002; Russell *et al.*, 2002). Perforin molecules can insert into the target cell membrane and polymerise, forming transmembrane pores (Podack *et al.*, 1984; Catalfamo *et al.*, 2003). Perforin pores contribute to osmotic lysis of cells, but perforin is mainly necessary for effective

granzyme-induced apoptosis, as illustrated by studies of apoptosis in perforin-deficient mice (Kagi *et al.*, 1994; Badovinac *et al.*, 2003). The exact role of perforin in granzyme-induced apoptosis remains to be determined, as it is debated whether perforin pores mediate granzyme entry into the target cell, or whether granzymes are endocytosed and perforin is involved in the release of granzymes from endosomes into the cytosol (Browne *et al.*, 1999; Shi *et al.*, 2005a). Granzyme B is the most studied granzyme (Lieberman, 2003). Evidence from various studies suggest that granzyme B may be able to enter target cells through receptor-dependent endocytosis (Shi *et al.*, 1997; Pinkoski *et al.*, 1998), but which receptor mediates uptake is controversial (Motyka *et al.*, 2000; Trapani *et al.*, 2003; Dressel *et al.*, 2004; Veugelers *et al.*, 2004). Granzyme A and granzyme B are the most abundant granzymes. They synergistically induce cell death through DNA degradation (Anel *et al.*, 1997; Beresford *et al.*, 1999; Fan *et al.*, 2003). Granzyme A attacks the integrity of the nuclear membrane, contributes to the opening of chromatin and activates a DNase that cuts into DNA single strands (Russell *et al.*, 2002; Lieberman *et al.*, 2003). Granzyme A also damages mitochondria, which releases pro-apoptotic factors (Martinvalet *et al.*, 2005). Granzyme B, either directly or through a protease cascade, activates a DNase that breaks DNA double strands (Lord *et al.*, 2003). Granzyme B also damages mitochondria. Other less well characterised granzymes participate in the induction of apoptosis (Grossman *et al.*, 2003).

CTL therefore produce various molecules that deliver signals precisely to target cells and induce cell death through numerous pathways, resulting in the coincidental killing of intracellular pathogen in a controlled manner.

$CD8^+$ T cell-mediated responses are indispensable for the control and clearing of many viral infections, such as infections with HIV (Matano *et al.*, 1998; Madden *et al.*, 2004), HSV (Simmons *et al.*, 1992), measles (Permar *et al.*, 2003), EBV (Rickinson *et al.*, 1997), bacterial infections with *Mycobacterium tuberculosis* (Flynn *et al.*, 1993; Smith *et al.*, 1999), *Listeria monocytogenes* (Ladel *et al.*, 1994), *Chlamydia pneumoniae* (Rottenberg *et al.*, 1999), fungal infections with *Histoplasma capsulatum* (Deepe, 1994), *Paracoccidioides brasiliensis* (Cano *et al.*, 2000), and parasitic infections with *Toxoplasma gondii* (Parker *et al.*, 1991; Shirahata *et al.*, 1994). Understanding what induces $CD8^+$ T cell effector functions, and how, is particularly important for the rational design of new safe vaccines. New vaccines are

indeed much needed against pathogenic intracellular organisms, such as HIV and *M. tuberculosis* for example, which have a heavy impact on public health and can be a major cause of death. Although live-attenuated viruses have been successfully used as vaccine agents, it is recognised that risks are associated with this approach. Live-attenuated organisms can behave as opportunistic pathogens in immunocompromised recipients, and may revert to pathogenic strains and cause disease in healthy recipients. Using recombinant protein antigens as vaccines is a safer option, and it is known that the induction of $CD8^+$ T cell responses is not restricted to antigens from infectious agents through direct priming, but cross-priming can also elicit functional responses, against exogenous antigens. Induction of cross-priming is a controlled process. Identifying the signals that initiate the induction of cross-priming, and studying the pathways that authorise cross-priming, will contribute to a better understanding of this process, and detailed knowledge on the induction of cross-priming is crucial to the design of potent and safe vaccines.

1.4 Research project objectives

A few microbial stimuli have been reported to induce cross-priming. The aim of this project was to assess the ability of a broader range of microbial stimuli to induce cross-priming, and to investigate the mechanisms by which they trigger this response. While it is important to ensure the generation of functional $CD8^+$ T cell responses, it is often desirable to enhance protective antibody responses. Therefore the effect of these microbial stimuli on the enhancement of immunoglobulin G responses was also assessed. The study was organised into three main parts:

- **Study of the effects of Toll-like receptor agonists on the enhancement of antibody responses and on the induction of cross-priming**

Microbial products have been shown to enhance antibody responses through activation of TLR signalling pathways. In addition, some of the microbial stimuli reported to induce cross-priming are TLR agonists, and a category of agonists was shown to induce cross-priming through their specific TLR. Therefore, the effects of representative agonists for a range of TLRs on the enhancement of immunoglobulin G responses and on the induction of cross-priming against model protein antigens were studied.

- **Study of the effects of carbohydrate microbial structures on adaptive immune responses**

Some biochemical structures are shared by organisms across kingdoms. High-mannose carbohydrate molecules are exposed by organisms as diverse as bacteria, viruses and fungi, and have been found to trigger responses from the adaptive immune system. Therefore, in order to further characterise the effect of high-mannose molecules on antigen-specific adaptive immune responses, their capacity to induce cross-priming, to polarise $CD4^+$ T cell responses and to enhance immunoglobulin G responses was assessed.

- **Investigation of the mechanisms of induction of cross-priming by microbial stimuli**

Particular signals, such as IFN-α/β and CD40/CD40L interactions, generated during the immune response have been shown to be able to control the licensing of cross-priming. The contribution of those signals to the induction of cross-priming by TLR agonists and high-mannose molecules was thus assessed. Since immune responses and cross-priming licensing signals can be initiated by the recognition of microbial stimuli by innate receptors, the involvement of relevant innate receptors in the induction of cross-priming by high-mannose carbohydrate molecules was investigated.

Chapter 2
Materials and methods

2.1 Animal model

Inbred mice of various strains were used as a mammalian model to study the effect of particular PAMPS on adaptive immune responses *in vivo*. All experiments followed protocols approved by the Ethical Review Committee of the Institute for Animal Health (IAH) and in accordance with the Animals (Scientific Procedures) Act 1986 and Home Office guidelines.

2.1.1 Mouse strains

Abbreviation	Strain name and characteristics	Supplier
B6	C57Bl/6 : CD45.2 (H-2^b)	Harlan: Harlan UK Ltd., Bicester, Oxfordshire, UK Institute for Animal Health specific pathogen-free (SPF) breeding unit: IAH, Compton, Berkshire, UK
B6 CD45.1	C57Bl/6: CD45.1 (H-2^b)	SPF
OT II	OT II: all $CD4^+$ T cells express a MHC II I-A^b-restricted $OVA_{323-339}$ -specific TCR (B6, CD45.2/$Rag1^{-/-}$ background)	SPF
129	129/SvEv (H-2^b)	SPF
IFNα/β$R^{-/-}$	IFN-α/β receptor-deficient (129 background)	SPF
MHC $II^{-/-}$	I-A-deficient (B6 background)	SPF
$CD40^{-/-}$	CD40-deficient: *Tnfrsf5*^{tm1Ki} targeted mutation (B6 background)	JAX® Mice: The Jackson Laboratory, Bar Harbor, Maine, USA Stock number 002928
IL-$12^{-/-}$	IL-12p35-deficient: *Il-12a*^{tm1Jm} targeted mutation (B6 background)	JAX® Mice Stock number 002692
B10	C57Bl/10ScSnJ: both B6 and B10 are sub-lines of the C57Bl line (H-2^b)	JAX® Mice Stock number 000476
$TLR4^{-/-}$	C57Bl/10ScNJ: TLR4-deficient (*tlr4*$^{lps-del}$ on B10 background).	JAX® Mice Stock number 003752

$MR^{-/-}$	Mannose receptor-deficient (B6 background)	Breeding pairs were a gift from Prof. Siamon Gordon (Sir William Dunn School of Pathology, Oxford University, UK). These mice originated from Prof. Michel C. Nussenzweig's laboratory (Laboratory of Molecular Immunology, Howard Hughes Medical Institute, The Rockefeller University, NY, USA). Mice were housed and bred at the IAH.

2.1.2 Injection protocols

Microlance™3 needle 30 gauge (GA)	BD Biosciences	304000
Microlance™3 needle 27 GA	BD Biosciences	302200
Microlance™3 needle 26 GA	BD Biosciences	303800

Route of injection	Site of injection	Sample volume	Needle GA
Intramuscular (i.m.)	Thigh of each leg	50 µL per leg	30
Intravenous (i.v.)	Tail vein	200 µL	27
Intraperitoneal	Abdomen	200 µL	26
Subcutaneous (s.c.)	Right flank	200 µL	26

2.2 Cell and blood sample preparation

2.2.1 General reagents and buffers

Red Blood Cell Lysing Buffer	Sigma	R 7757
RPMI 1640 medium with GlutaMAX™, 25 mM HEPES	Invitrogen (Gibco)	72400
Foetal bovine serum (FBS)	Invitrogen (Gibco)	10106 Batch 40G2027K
Phosphate-buffered saline (PBS) pH 7.4 (10×) (10 mM KH_2PO_4, 1.54 M NaCl, 30 mM $Na_2HPO_4.7H_2O$)	Invitrogen (Gibco)	14200
Distilled water (dH_2O)	Baxter	UKF7114

2.2.1.1 RF10 buffer (RPMI, 10% FBS)

RPMI 1640	500 mL
FBS (heat inactivated)	50 mL

2.2.1.2 Wash buffer RF5 (RPMI, 5% FBS)

RPMI 1640	500 mL
FBS (heat inactivated)	25 mL

2.2.1.3 PBS

PBS 10×	100 mL
dH_2O	add to 1 L

2.2.2 Preparation of single-cell suspensions

Frosted microscope slides	Erie Scientific	2951
Petri dishes	Bibby Sterilin	101RT
Cell strainers 45μm	BD Falcon™	352340
50 mL conical tubes	BD Falcon™	352070
Improved Neubauer counting chamber	Weber Scientific International	AS 1000
Red Blood Cell lysing buffer	Sigma	R 7757
Trypan blue stock solution (0.4%)	Sigma	T 8154

To prepare a single-cell suspension, lymphoid organs collected from mice were gently mashed between frosted slides in a Petri dish in RF5 medium (see 2.2.1). The cell suspension was filtered through a cell strainer, centrifuged at 300 × g for 7 min at 4°C, and red blood cells were lysed in ammonium chloride buffer for 3 min on ice. Cells were washed and live cells were counted in trypan blue 0.1%; trypan blue is an exclusion dye that stains dead cells violet-blue.

2.2.3 $CD8^+$ T cell enrichment

Anti-mouse CD4 hybridoma supernatant (clone GK1.5)	In-house preparation (Memory Group, EJIVR)	
Anti-mouse MHC II hybridoma supernatant (clone M5/114.15.2)	In-house preparation (Memory Group, EJIVR)	
Anti-mouse B220 hybridoma supernatant (clone RA36B2)	In-house preparation (Memory Group, EJIVR)	
Dynabeads® M-450 sheep anti-rat IgG	Dynal Biotech	110.08
Dynabeads® M-450 sheep anti-mouse IgG	Dynal Biotech	110.02

Enriched $CD8^+$ T cell were obtained by negative selection using magnetic Dynabeads. Splenic single-cell suspensions were pelleted and resuspended in a cocktail of anti-CD4, anti-MHC II and anti-B220 antibodies. After 15 min incubation on ice, cells were washed and resuspended in RF10 (see 2.2.1) containing a mixture of anti-rat and anti-mouse IgG Dynabeads. After 40 min incubation at 4°C with continuous rotation, samples were placed against a cell separation magnet and supernatants, enriched in $CD8^+$ T cells, were collected for further use.

2.2.4 $CD4^+$ T cell enrichment

Anti-mouse CD8 hybridoma supernatant (clone 53-6.72)	In-house preparation (Memory Group, EJIVR)	
Anti-mouse MHC II hybridoma supernatant (clone M5/114.15.2)	In-house preparation (Memory Group, EJIVR)	
Anti-mouse B220 hybridoma supernatant (clone RA36B2)	In-house preparation (Memory Group, EJIVR)	
Dynabeads® M-450 sheep anti-rat IgG	Dynal Biotech	110.08
Dynabeads® M-450 sheep anti-mouse IgG	Dynal Biotech	110.02

As for $CD8^+$ T cell enrichment, $CD4^+$ T cell were purified by negative selection, using a cocktail of anti-CD8, anti-MHC II and anti-B220 antibodies followed by magnetic separation.

2.2.5 Preparation of antigen presenting cells

2.2.5.1 Reagents and culture medium

2-Mercaptoethanol, 50 mM	Invitrogen (Gibco)	31350
Penicillin (10,000 units/mL) / streptomycin (10,000 µg/mL)	Invitrogen (Gibco)	15140
Anti-mouse Thy-1.1,1.2 hybridoma supernatant (clone T24.2)	In-house preparation (Memory Group, EJIVR)	
Guinea-pig complement	Cedarlane	CL4051

RF10 growth medium (RPMI, 10% FBS)

RPMI 1640	500 mL
FBS (heat inactivated)	50 mL
2-Mercaptoethanol 50 mM Final concentration is 50 µM	0.5 mL
Penicillin (10,000 units/mL) / streptomycin (10,000 µg/mL) Final concentration is penicillin (100 units/mL) / streptomycin (100 µg/mL)	5 mL

2.2.5.2 T-cell depletion

For enrichment in antigen-presenting cells, splenic single-cell suspensions from naïve mice were depleted of T-cells by antibody-directed complement lysis. Cells were incubated with anti-Thy-1.2 antibodies, in the presence of complement, for 1 h at 37°C. Cells were filtered, washed and irradiated at 3,000 rad. Cells were then filtered, washed, counted and diluted to the appropriate concentration in RF10 growth medium.

2.2.6 Separation of mononuclear cells from blood

Heparin (1,000 IU/mL)	Leo Laboratories	
Histopaque®-1083	Sigma	1083-1

Blood samples were collected on heparin (50 IU/mL final concentration), diluted 1:1 in PBS and under layered with the polysucrose gradient medium Histopaque®-1083 (density 1.083 g/mL). Mononuclear cells were isolated by density gradient centrifugation at 460 × g for 20 min at RT, with the centrifuge brakes switched off. The layer of mononuclear cells, situated on top of the gradient medium, was collected and cells were washed twice in PBS.

2.2.7 Separation of serum from blood

Blood samples were collected from mice by tail-vein bleeding. Serum was separated from clotted blood by centrifugation at 17,530 × g for 20 min at 4°C. Sera were stored at -20°C.

2.3 Model proteins and PAMPS

2.3.1 Proteins

Ovalbumin (OVA) grade VI	Sigma	A 2512
Chicken γ globulin (CGG)	Jackson ImunoResearch Laboratories	003-000-0020

2.3.2 TLR agonists

Peptidoglycan from *Staphylococcus aureus*	TLR2/X	InvivoGen	tlrl-pgn
Zymosan from *Saccharomyces cerevisiae*	TLR2/6	InvivoGen	tlrl-zyn

Polyinosinic-polycytidylic acid (poly I:C)	TLR3	Sigma	P 1530
Lipopolysaccharide (LPS) from *Escherichia coli*	TLR4	Sigma	L 4524
R-848	TLR7/8	InvivoGen	tlrl-r848
CpG 2216 5'-ggG GGA CGA TCG TCg ggg gg-3'	TLR9	MWG Biotech	Custom synthesis
CpG 2216 control 5'-ggG GGA GCA TGC TGC ggg gg-3'		MWG Biotech	Custom synthesis
CpG 1668 5'-tcc atg acg ttc ctg atg ct-3'	TLR9	MWG Biotech	Custom synthesis
CpG 1720 (CpG 1668 control) 5'-tcc atg agc ttc ctg atg ct-3'		MWG Biotech	Custom synthesis

2.3.3 Glycolipids

Escherichia coli O9:K9(L9):H12 (*E. coli* O9)	National Collection of Type Cultures (NCTC)	9009 Bi 316/42
LPS (*E. coli* O55:B5)	Sigma	L 4524
LPS (*Porphyromonas gingivalis*)	InvivoGen	tlrl-pglps

LPS from *Klebsiella pneumoniae* O3:K55 and *K. pneumoniae* K52 were obtained from Dr Susanne Zamze (Carbohydrate Immunology Group, EJIVR). Lipooligosaccharides (LOS) from *Neisseria meningitidis* 44/76 and *N. meningitidis* 44/76 mutant 4 were obtained from Dr Graeme Frith (Carbohydrate Immunology Group, EJIVR). LPS and LOS were extracted from bacteria by the hot phenol-water method.

2.3.3.1 Bacteria culture and extraction of lipopolysaccharide

Luria-Bertani (LB) broth	Media Services, IAH	
LB agar	Media Services, IAH	
Phenol	Sigma	P 1037
Molecularporous membrane tubing 12,000-14,000 molecular weight cut-off (MWCO)	Spectrum	132 700

A sample of freeze-dried *E. coli* O9 was rehydrated in LB medium according to NCTC recommendations, plated onto LB agar and incubated overnight (O/N) at 37°C. A colony was transferred to 20 mL LB broth to make a starter-culture. After incubation O/N at 37°C at 0.4 × g in a shaker, 2.5 mL of starter-culture were cultured in 4 L of LB broth O/N at 37°C at 0.3 × g. The bacterial suspension was centrifuged

at 10,000 × g for 30 min at 4°C. The pellet was resuspended in 50 mL PBS and LPS was extracted with hot 45% aqueous phenol: 50 mL of saturated aqueous phenol (90% (w/v)) were heated to 65°C and then added to the equal volume of pre-heated bacterial suspension. The suspension was stirred at 65°C for 15 min. Phase separation was obtained by cooling the preparation on ice before centrifugation at 230 × g for 15 min at 4°C. The aqueous phase, which contains LPS, was collected. LPS extraction from the preparation was repeated, after replacing the aqueous phase by an equal volume of water. The aqueous phases from both extractions were combined and dialysed extensively against dH_2O, using 12-14,000 MWCO dialysis tubing. After dialysis, the solution was ultra-centrifuged at 100,000 × g at 4°C O/N, and the LPS pellet was resuspended in dH_2O.

2.3.3.2 Purification of glycolipids

Phenol-extracted LPS and LOS and commercial LPSs were further purified by size exclusion chromatography in a detergent-containing buffer, to remove LPS- and LOS-associated proteins (Andersen *et al.*, 2002).

2.3.3.2.1 Reagents and buffers

HiLoad 26/60 Superdex 75 gel filtration column	Amersham Biosciences	17-1070-01
Hiload 26/60 Superdex 200 gel filtration column	Amersham Biosciences	17-1071-01
TSKgel G5000PW gel filtration column	Tosoh Bioscience	05764
Stericup™ Filter unit 0.22 µm	Millipore	SCGPU10RE
Molecularporous membrane tubing 6,000-8,000 molecular weight cut-off (MWCO)	Spectrum	132 650
Sodium deoxycholate	Sigma	D 5670
Tris-(hydroxymethyl)-aminomethane (Tris)	Sigma (Riedel de Haën)	33742
Hydrochloric acid (HCl) 7M	VWR (BDH)	18036 5D
Ethylenediaminetetraacetic acid (EDTA)	Sigma	E 5134
Sodium chloride (NaCl)	VWR (BDH)	443827W
Endotoxin-free water	InvivoGen	Tlrl-h2olal25
Ethanol	VWR (BDH)	10107

Sodium deoxycholate (NaDOC) dissociating buffer (20 mM Tris / HCl, pH 8.5, 2 mM EDTA, 0.154 M NaCl and 1% (w/v) NaDOC)

Tris	2.423 g
pH was adjusted to 8.5 with HCl	
EDTA	0.744 g
NaCl	9.009 g

dH_2O add to 1 L

The solution was filtered through a 0.22 μm membrane, using a Stericup™ filter unit, before the addition of NaDOC.

NaDOC 10 g

2.3.3.2.2 Preparative high performance liquid chromatography (HPLC) and ethanol- precipitation

Glycolipid samples were solubilised in NaDOC buffer and injected into a Superdex 75, a Superdex 200 or a TSK G5000 gel filtration column. The refractive index (RI) of the eluant and absorbance at 280 nm were monitored. This method allowed for proteins and nucleic acid contaminants to elute separately from LPS and LOS, which were detected by an increased RI. Glycolipid-containing fractions were pooled and dialysed extensively at RT against dH_2O, using 6-8,000 MWCO dialysis tubing. After dialysis, samples were freeze-dried. LPS and LOS were precipitated by addition of 0.2 M NaCl and ice-cold 80% (v/v) ethanol (final concentration). LPS and LOS pellets were recovered after centrifugation, left to dry and resuspended in 1 mL of endotoxin-free water.

2.3.3.3 Characterisation of the glycolipid preparations

Purified samples were analysed by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS), and LPS and LOS were visualised by silver staining.

2.3.3.3.1 SDS-PAGE

Mini-PROTEAN II electrophoresis Module	Bio-Rad	165-2944
30% Acrylamide/bisacrylamide solution 29:1	Bio-Rad	161-0156
Tris	Bio-Rad	161-0716
SDS	Invitrogen (Gibco BRL)	15525-025
Ammonium persulfate	Amersham Biosciences	76322
N,N,N',N'-tetra-methyl-ethylenediamine (TEMED)	Bio-Rad	161-0800
Laemmli sample buffer	Bio-Rad	161-0737
2-mercapthoethanol	Sigma	M 3148
Tris/glycine/SDS buffer 10× (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3)	Bio-Rad	161-0732
Perfect Protein™ molecular weight markers 15-150 kDa	Novagen	69149-3

Buffers and gels were prepared according to (Sambrook *et al.*, 1989a)

Resolving gel buffer (1.5 M Tris-HCl pH 8.8 and 0.1% SDS)

Tris	18.17 g
pH was adjusted to 8.8 with HCl	
SDS 10% (w/v)	1 mL
dH_2O	add to 100 mL

12% polyacrylamide resolving gel

dH_2O	3.3 mL
30% Acrylamide / bisacrylamide solution	4.0 mL
1.5 M Tris (pH 8.8)	2.5 mL
SDS 10%	0.1 mL
Ammonium persulfate 10% (w/v)	0.1 mL
TEMED	0.004 mL

Stacking gel buffer (0.5 M Tris-HCl pH 6.8 and 0.1% SDS)

Tris	6.06 g
pH was adjusted to 6.8 with HCl	
SDS 10% (w/v)	1 mL
dH_2O	add to 100 mL

5% polyacrylamide stacking gel

dH_2O	3.4 mL
30% Acrylamide / bisacrylamide solution	0.83 mL
0.5 M Tris (pH 6.8)	0.63 mL
SDS 10%	0.05 mL
Ammonium persulfate 10% (w/v)	0.05 mL
TEMED	0.005 mL

SDS-PAGE gels were casted between glass plates (inner plate, 102×82 mm, and outer plate, 102×73 mm). Once the resolving gel was set, the stacking gel was poured on top and wells were moulded with a Teflon comb. Gels were mounted onto the electrode clamping frame, in the electrophoresis tank containing Tris/glycine/SDS running buffer. Glycolipid samples were diluted 1:2 in reducing sample buffer (Laemmli buffer, 5% (w/v) 2-mercaptoethanol), then heated for 3 min in boiling water and deposited into wells in the gel. A 25 mA current was applied between the electrodes until the dye front had moved into the resolving gel, then the current was increased to 40 mA. The electrophoresis process was stopped when the dye front reached the bottom of the resolving gel.

2.3.3.3.2 Silver staining of SDS-PAGE gels

Silver staining was performed as described by (Tsai *et al.*, 1982)

Acetic acid (CH_3COOH)	VWR (BDH)	10001CU
Periodic acid ($IO_6 H_5$)	Sigma	P-5463
Sodium hydroxide (NaOH)	Sigma	S-8045
Ammonium hydroxide (NH_4OH) 28%	Sigma	A-6899
Silver nitrate ($AgNO_3$)	Sigma	S-6506
Sodium citrate, tribasic	Invitrogen (Gibco BRL)	15584-022
Formaldehyde 37%	Sigma (Aldrich)	25,254-9
Methanol	Sigma (Fluka)	65543

Fixing solution (40% ethanol, 5% acetic acid)

Ethanol	200 mL
Acetic Acid	25 mL
dH_20	add to 500 mL

Oxidising solution (0.7% (w/v) periodic acid in fixing solution)

Periodic acid	0.7 g
Fixing solution	100 mL

Silver staining reagent (0.67% (w/v) $AgNO_3$)

0.1 M NaOH (0.4% (w/v))	14 mL
NH_4OH	1 mL
$AgNO_3$	0.5 g
dH_20	58 mL

Developing solution

Sodium citrate	50 mg
Formaldehyde	0.5 mL
dH_20	1 L

Glycolipids were fixed in the polyacrylamide gel using fixing solution. Gels were incubated for 1 h with gentle agitation on a rotating rocker. The fixing solution was replaced with 50 mL of oxidising solution for 5 min. After three 30 min washes with dH_20, freshly prepared silver staining reagent was poured and gels were incubated for 10 min. After three 10 min washes with dH_20, gels were developed until desired band intensity was obtained. The reaction was then quenched with 50% methanol for a few minutes. Gels were stored in dH_20.

SDS-PAGE gel drying

DryEase® Mini-gel drying system	Invitrogen	N12387
Glycerol	Invitrogen (Gibco BRL)	15514-011

Drying solution (30% ethanol, 5% glycerol)

Ethanol	150 mL
Glycerol	25 mL
dH_2O	add to 500 mL

Stained gels were equilibrated in drying solution, according to manufacturer's instructions, placed between two sheets of cellophane previously wetted with drying solution, and mounted onto a frame to dry.

2.3.3.3.3 Glycolipid quantification

Sodium (meta)periodate ($NaIO_4$)	Sigma (Fluka)	71859
Purpald (4-Amino-5-hydrazino-1,2,4-triazole-3-thiol)	Sigma (Aldrich)	16,289-2
LPS (*E. coli*)	Sigma	L 4524
LPS (*N. meningitidis*) 2mg/mL	In house-preparation (Dr S. R. Andersen, G. R. Guile) (Andersen *et al.*, 2002)	

Sodium periodate 32 mM

$NaIO_4$	684.5 mg
dH_2O	100 mL

Sodium periodate 64 mM

$NaIO_4$	1.369 g
dH_2O	100 mL

Purpald reagent 136 mM in 2N NaOH

NaOH	800 mg
dH_2O	10 mL
Purpald	199 mg

Concentrations of the purified glycolipids were determined by comparison with LPS standards using the purpald colorimetric assay, as described by (Lee *et al.*, 1999). In a 96-well plate, 50 µL samples were oxidised for 25 min with 50 µL of 32 mM $NaIO_4$, producing formaldehyde. Addition of 50 µL of 136 mM purpald reagent led to a reaction with formaldehyde. The resulting colourless product was converted to a purple chromogene by oxidisation for 20 min with 50 µL of 64 mM $NaIO_4$. Absorbance was measured at 550 nm.

2.3.4 Mannan and high-mannose structures

2.3.4.1 Preparation of modified-mannan ovalbumin conjugates

Mannan from *Saccharomyces cerevisiae*	Sigma (Fluka)	63557
Sodium phosphate, dibasic (Na_2HPO_4)	Sigma	S-9763
Sodium phosphate, monobasic ($NaH_2PO_4.H_2O$)	Sigma	S-3522
Sodium bicarbonate ($NaHCO_3$)	VWR (BDH)	102475W
Sodium borohydride (BH_4Na)	Sigma (Fluka)	71321
Ethylene glycol ($C_2H_6O_2$)	Sigma	E-9129
PD-10 desalting columns	Amersham Biosciences	17-0851-01
Micro BCA™ Protein Assay Kit	Pierce	23235

2.3.4.1.1 Mannan oxidisation, conjugation to protein and reduction of conjugates

From (Apostolopoulos *et al.*, 1995)

0.1 M phosphate buffer, pH 6.0
From (Sambrook *et al.*, 1989b)

Na_2HPO_4 (in 12 mL dH_2O)	1.704 g
NaH_2PO_4. H2O (in 88 mL dH_2O)	12.144 g

0.1 M bicarbonate solution, pH 8.0-9.0

$NaHCO_3$	8.4 g
dH_2O	100 mL

Mannan, at 14 mg/mL in 0.1 M phosphate buffer pH 6.0, was oxidised with 0.02 M sodium periodate (final concentration) for 60 min at 4°C. The reaction was quenched with 10 µL ethylene glycol/mL of solution, and the oxidised mannan was separated through a PD-10 desalting column equilibrated with bicarbonate solution, pH 8-9. Oxidised mannan, eluted in the 2 mL void volume, was mixed with 2.5 mg ovalbumin and incubated O/N at RT.

Reduced-mannan ovalbumin conjugates (red-M Ova) were obtained by treating oxidised-mannan conjugates (ox-M Ova) with 1 mg/mL sodium borohydride for 3 h at RT. Conjugates were used without further purification, and OVA contents were verified using a protein assay, based on the bicinchoninic acid (BCA) colorimetric assay.

2.3.4.2 *Limulus* amoebocyte lysate assay

Limulus amebocyte lysate (LAL) ENDOSAFE® kinetic turbidimetric assay	Charles River ENDOSAFE	
LAL reagent water	Charles River ENDOSAFE	WL110
Depyrogenated glass test tubes	Charles River ENDOSAFE	TL700
Depyrogenated pipettes, 1 mL	Charles River ENDOSAFE	PL100
Depyrogenated pipettes, 5 mL	Charles River ENDOSAFE	PL500
Microtest 96-well plates, non pyrogenic	BD Falcon™	353072

Presence of endotoxin in mannan samples was determined using the LAL assay. LAL reagents are prepared from clotting enzymes contained in horseshoe crab blood granules. Endotoxin activates the *Limulus* compounds, which induce gelation and increased opacity of the solution tested. The LAL assay was performed in a 96-well microplate and was standardised with control endotoxin from *E. coli* O55:B5, provided by the manufacturer. Pyrogen-free water was used as a negative control. After addition of LAL reagent to samples and controls, absorbance was read at 37°C, every 30 s for 1 h at 349 nm, to determine the kinetics of clotting. Endotoxin values were calculated in reference to the *E. coli* endotoxin onset-time standard curve, using the SOFTMax® PRO Kinetic Turbidimetric Protocol with a SPECTRAmax® Microplate Spectrophotometer.

2.3.4.3 Preparation of bromelain-cleaved hemagglutinin from *Influenza*

2.3.4.3.1 Reagents and buffer

Influenza X:31, A/Aichi/68 (H3/N2)	Charles River Laboratories (SPAFAS)	490715
Bromelain	VWR (Calbiochem)	203761
Tris HCl	Sigma	T 3253
EDTA	Sigma	E 5134
2-mercapthoethanol	Sigma	M 3148
NaCl	VWR (BDH)	443827W
Slide-A-Lyser® dialysis cassette (10,000 MWCO)	Perbio (Pierce)	66380

TEM buffer (0.1 M Tris HCl pH 8.0, 1.3 mM EDTA and 0.05 M mercaptoethanol)

Tris HCl	1.576 g
pH was adjusted to 8.0	
EDTA	48.4 mg
Mercaptoethanol	351 µL
dH2O	add to 100 mL

2.3.4.3.2 Bromelain-cleaved hemagglutinin

Hemagglutinin (HA) was released from Influenza X:31 by digestion with bromelain as described by (Brand *et al.*, 1972; Bonnafous *et al.*, 2000), with slight modifications. A virus sample (1 mL at 2 mg of protein per mL) was thawed on ice and pelleted by centrifugation at 55,000 × g for 1 h 30 at 4°C. The pellet was soaked in 1 mL TEM buffer for 3 h at RT and then resuspended. Bromelain was added at virus/enzyme 2:1 (w/w). After incubation for 17 h at 37°C, the viral cores were pelleted by centrifugation at 55,000 × g for 40 min at 4°C, and HA was collected from the supernatant. The HA preparation was dialysed against PBS using a 10,000 MWCO dialysis cassette.

2.4 Measure of cytokine concentration in serum and cell culture supernatant

2.4.1 Flow cytometry multiplex assays

Cytometric Bead Array (CBA) Mouse Inflammation	BD Biosciences	552364
Mouse Th1/Th2 cytokine CBA	BD Biosciences	551287

Concentration of various immunoregulatory cytokines was measured using flow cytometry arrays. These assays are based on cytokines being captured with beads that are coated with antibodies specific for different cytokines. The antibodies have discrete fluorescence intensities, specific for each cytokine tested and detectable in the FL3 channel. Mixed bead populations were incubated with serum samples or tissue culture supernatants, in the presence of cytokine detection-antibodies conjugated to R-phycoerythrin (PE), which is detectable in the FL2 channel. Capture beads, cytokines and detection antibodies formed sandwich complexes; fluorescence in FL2 channel was proportional to the quantity of cytokine. Standard curves of FL2 fluorescence, generated with a range of known quantities of recombinant cytokines, allowed for calculations of the concentration of all cytokines tested in the sample.

2.4.2 Interferon-α ELISA

Mouse Interferon Alpha (Mu-IFN-α) ELISA kit	PBL Biomedical Laboratories	42100-1

The kit is based on a sandwich ELISA. IFN-α from serum samples was captured first by an anti-mouse IFN-α antibody in pre-coated 96-well plates. IFN-α, thus retained on the plate after washing, was then detected by a second anti-mouse IFN-α antibody conjugated to horseradish peroxidase (HRP). Addition of tetramethyl-benzidine (TMB), a HRP substrate, resulted in the formation of a chromogene, which absorbance was measured at 450 nm. Concentration of IFN-α in samples was calculated in reference to a standard curve.

2.5 Characterisation of immunoglobulin responses

2.5.1 Reagents and buffers

96-well flexible plate	BD Falcon™	353912
Sodium bicarbonate ($NaHCO_3$)	VWR (BDH)	102475W
Sodium carbonate (Na_2CO_3)	VWR (BDH)	102405Y
Dried skimmed milk	MARVEL	
Tween 20	Sigma	P 7949
Phosphate-buffered saline (PBS) pH 7.4 (10×) (10 mM KH_2PO_4, 1.54 M NaCl, 30 mM $Na_2HPO_4.7H_2O$)	Invitrogen (Gibco)	14200
Biotin-conjugated rat anti-mouse IgG1 monoclonal antibody	BD (Pharmingen)	553441
Biotin-conjugated rat anti-mouse IgG2a monoclonal antibody	BD (Pharmingen)	553388
Biotin-conjugated rat anti-mouse IgG2b monoclonal antibody	BD (Pharmingen)	553393
Biotin-conjugated rat anti-mouse IgG3 monoclonal antibody	BD (Pharmingen)	553401
Streptavidin-horseradish peroxidase (SAv-HRP) conjugate	BD (Pharmingen	554066
o-Phenylenediamine dihydrochloride (OPD) tablets	Sigma	P 9187
Hydrochloric acid (HCl) 37%	VWR (BDH)	101254H

2.5.1.1 ELISA coating buffer (carbonate buffer, pH 9.6)

2.5.1.1.1 1 M $NaHCO_3$

$NaHCO_3$	8.4 g
dH_2O	100 mL

2.5.1.1.2 Coating buffer

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
pH was adjusted to 9.6 with 1 M $NaHCO_3$	
dH_2O	add to 1 L

2.5.1.2 Blocking solution (PBS, 4% (w/v) dried milk)

Dried milk	6 g
PBS	150 mL

2.5.1.3 Serum dilution solution (PBS, 1% (w/v) dried milk, 0.2% Tween)

Dried milk	1.5 g
Tween 20	0.3 mL
PBS	add to 150 mL

2.5.1.4 Detection-antibody dilution solution (PBS, 1% (w/v) dried milk)

Dried milk	2.5 g
PBS	250 mL

2.5.1.5 Wash buffer (PBS, 0.05% Tween)

Tween 20	2.5 mL
PBS	5 L

2.5.1.6 Stop solution (3M HCl)

dH_20	374.5 mL
HCl	125.4 mL

2.5.2 ELISA protocol

CGG-specific serum antibody titres were measured using ELISA ten days after i.m. immunisation. ELISA plates were coated with 100 µL/well of 5 µg/mL CGG (see 2.3.1) in carbonate buffer and incubated O/N at RT. The coating solution was replaced with 150 µL/well of blocking solution to prevent non-specific binding of antibodies to plastic, and plates were incubated for 45 min at 37°C. Plates were washed three times and 100 µL/well of sera, in twelve two-fold serial dilutions, were transferred to the plates and incubated for 1 h at RT, to allow anti-CGG antibodies to bind to the antigen. Plates were washed three times. CGG-specific antibody isotypes were detected with 100 µL/well of biotinylated rat anti-mouse Ig antibodies for 1h at RT. After three washes, 100 µL/well of HRP-conjugated streptavidin were added for 1h at RT, to bind to biotin and amplify the detection of CGG-specific antibodies. Plates were washed three times and OPD, a HRP substrate, was added at 100 µL/well to induce a chromogenic reaction. Colour development was stopped by addition of 50 µL of 3 M HCl before the highest dilution of the highest titre serum rose above background. Absorbance was measured at 492 nm. Results were expressed as reciprocal endpoint titres, which were determined as first dilution below an arbitrary threshold of positivity for optical densities (OD). The threshold of positivity was

calculated for each antibody isotype as the average + 3 SD of all dilutions from three control mouse sera (i.e. sera from mice injected with PBS).

2.6 Characterisation of antigen-specific $CD8^+$ T cell responses

2.6.1 Tetramer and intracellular granzyme B staining

2.6.1.1 Reagents and buffers

Cyanine-5-conjugated anti-mouse CD8a antibody (anti-CD8-Cy5; clone YTS.169)	In-house preparation (Memory Group, EJIVR)	
Fluorescein isothiocyanate-conjugated anti-mouse CD8a antibody (anti-CD8-FITC)	BD (Pharmingen)	553031
R-phycoerythrin-conjugated H-2K^b-SIINFEKL tetramers (Tet-PE)	ProImmune	
Allophycocyanin-conjugated H-2K^b-SIINFEKL tetramers (Tet-APC)	ProImmune	
APC-conjugated anti-human granzyme B antibody	Caltag Laboratories	MHGB05
APC-conjugated mouse IgG1 isotype control	Caltag Laboratories	MG105
Sodium azide (NaN_3)	Sigma	S 8032
Paraformaldehyde	Sigma	P 6148
Saponin	Sigma	S 7900

2.6.1.1.1 Fluorescence-activated cell sorting (FACS) buffer (PBS, 2% FBS, 0.1% (w/v) azide)

PBS 10×	100 mL
FBS (heat inactivated)	20 mL
Sodium azide	1 g
dH_2O	add to 1 L

2.6.1.1.2 Fixing stock solution (PBS, 4% (w/v) paraformaldehyde, pH 7.4)

Paraformaldehyde	2 g
A couple of drops, from a glass Pasteur pipette, of concentrated NaOH were added to help paraformaldehyde dissolve, then pH was adjusted to 7.4 with HCl	
PBS	50 mL

2.6.1.1.3 Permeabilisation buffer (FACS buffer, 0.1% (w/v) saponin)

Saponin	10 mg
FACS buffer	10 mL

2.6.1.2 Staining procedures

Eight days after i.m. immunisation, spleens were removed and $CD8^+$ T cells were purified from single-cell suspensions (see 2.2.2 and 2.2.3). Alternatively, blood samples were collected from immunised mice at day eight and mononuclear cells were purified by density gradient centrifugation (see 2.2.6).

For tetramer staining, cells were incubated for 30 min at RT with Tet-PE first, washed twice with FACS buffer, then stained for 10 min at RT with anti-CD8-Cy5. Alternatively, cells were stained with Tet-APC and anti-CD8-FITC. Labelled cells were washed twice before being analysed by flow cytometry.

For intracellular granzyme B staining, cells were labelled with anti-CD8-FITC and Tet-PE, and fixed with 2 % paraformaldehyde in FACS buffer for 20 min on ice. After one wash, cells were permeabilised for 10 min at RT, and then incubated for 30 min at RT with anti-granzyme B-APC or isotype control-APC (Wherry *et al.*, 2003); both antibodies were in solution in permeabilisation buffer. Labelled cells were washed twice before being analysed by flow cytometry.

All FACS acquisitions were done on a Becton Dickinson FACScalibur and all data were analysed using the CellQuest Pro software from BD Biosciences.

2.6.2 IFN-γ ELISPOT

Multiscreen™ plate	Millipore	MAHAS4510
Purified rat anti-mouse IFN-γ monoclonal antibody	BD (Pharmingen)	551216
Biotinylated rat anti-mouse IFN-γ monoclonal antibody	BD (Pharmingen)	554410
Alkaline phosphatase-conjugated goat anti-biotin antibody	Vector Laboratories	SP-3020
Alkaline phosphatase conjugate substrate kit	Bio-Rad	170-6432
$OVA_{(257-264)}$ peptide (SIINFEKL)	Custom synthesis	IAH

Mixed cellulose-ester membrane plates were coated with anti-IFN-γ capture antibody at 5 μg/mL in carbonate buffer (see 2.5.1) and incubated O/N at 4°C. $CD8^+$ T cells purified from immunised mice (see 2.6.1.2) were seeded in duplicate on the plates, at 1×10^5 cells/well in RF10 growth medium, and incubated for 36 h with 5×10^5 cells/well syngeneic splenocytes (see 2.2.4), in the presence or absence of 1 μM SIINFEKL peptide. Plates were washed eight times with PBS, then with PBS/0.05%

Tween 20, and captured-IFN-γ was detected by adding biotinylated anti-IFN-γ antibody for 2 h at 37°C. After eight washes with PBS/0.05% Tween 20, the detection step was amplified by an alkaline phosphatase-conjugated anti-biotin antibody incubated for 2 h at 37°C. IFN-γ spots were visualised on the membrane of the plates by adding 5-bromo-4-chloro-3-indolyl phosphate, a substrate for alkaline phosphatase, in nitroblue tetrazolium solution. The reaction formed a purple precipitate at the site where cells had produced IFN-γ. Spots were counted under an inverted-light microscope.

2.6.3 *In vivo* cytotoxic T lymphocyte assay

2.6.3.1 Reagents and buffers

Bovine serum albumin (BSA)	Sigma	A 4503
5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE)	Molecular Probes	C-1157

2.6.3.1.1 PBS, 5% FCS

FBS (heat inactivated)	50 mL
PBS	add to 1 L

2.6.3.1.2 PBS, 0.1% (w/v) BSA

BSA	1 g
PBS	1 L

2.6.3.2 Cytotoxicity assay

The assay was adapted from methods described by (Aichele *et al.*, 1997; Oehen *et al.*, 1998). OVA-specific cytotoxic activity was assessed by measuring the killing of target splenocytes pulsed with SIINFEKL. A suspension of naïve splenocytes was prepared at 2×10^7 cells/mL in RF5 medium and divided into two populations. The target population was pulsed with 1 nM SIINFEKL for 1 h at 37°C, while the internal control population was not pulsed with peptide. Both populations were then washed twice with PBS, 0.1% BSA, and diluted to 1×10^7 cells/mL in PBS, 0.1% BSA. Target cells were labelled with a low concentration of CFSE (0.1 μM; $CFSE^{low}$), and unpulsed cells with a higher concentration of CFSE (1μM; $CFSE^{high}$). Cells were stained for 7 min at 37°C before the reaction was quenched by diluting each population into 5 volumes of ice-cold PBS, 5% FCS. Cells were filtered, washed three times in PBS and counted. Target and control cells were mixed 1:1 in

PBS and 2×10^7 cells were injected i.v. into syngeneic mice immunised nine days earlier. Eighteen hours later, spleens from immunised mice were recovered and single-cell suspensions were analysed by flow cytometry to measure the proportion of remaining $CFSE^{low}$ cells versus $CFSE^{high}$. A reduction in the proportion of $CFSE^{low}$ cells, compared to percentages of $CFSE^{low}$ and $CFSE^{high}$ cells in control mice, represented SIINFEKL-specific lysis of target cells. Specific lysis was calculated as follows:

$$\% \quad \text{specific} \quad \text{lysis} = \left[1 - \left(\frac{\%CFSE^{low}\ \text{sample}}{\%CFSE^{high}\ \text{sample}} \div \frac{\%CFSE^{low}\ \text{control}}{\%CFSE^{high}\ \text{control}}\right)\right] \times 100.$$

When comparing cytotoxic function between two mouse strains, such as wild-type (WT) and knock-out (KO), results were sometimes expressed as a reduction in specific lysis in KO mice:

$$\%\ \text{reduction} = \left[1 - \left(\frac{\%\,\text{SIINFEKL - specific lysis in KO mouse}}{\text{mean}\,\%\,\text{SIINFEKL - specific lysis in 3 WT mice}}\right)\right] \times 100$$

2.7 Characterisation of antigen-specific $CD4^+$ T cell responses

Mice were immunised subcutaneously with OVA ± adjuvant and were injected intravenously with 1.5×10^6 OVA-specific $CD4^+$ T cells from lymph nodes of OT II mice. Ten days after immunisation, draining inguinal lymph nodes were collected and single cell suspensions were prepared. Some cells were used to measure proliferation of antigen-specific $CD4^+$ T cell, while the rest was used to quantify cytokines produced after re-stimulation *in vitro*.

2.7.1 Measure of proliferation *in vivo*

Cyanine-5-conjugated anti-mouse CD4 antibody (anti-CD4-Cy5; clone GK1.5)	In-house preparation (Memory Group, EJIVR)	
FITC-conjugated mouse anti-mouse CD45.2 monoclonal antibody (anti-CD45.2-FITC)	BD (Pharmingen)	553772

To characterise proliferation of $CD4^+$ OVA-specific OT II cells, $CD4^+$ T cells were purified from lymph nodes (see 2.2.4), then labelled with anti-CD4-Cy5 and anti-CD45.2-FITC in FACS buffer for 10 min at RT. Cells were washed and analysed by

flow cytometry. Numbers of OVA-specific $CD4^+$ T cells per draining lymph node (dLN) were calculated by multiplying the percentage of $CD45.2^+$ $CD4^+$ cells by the total number of dLN cells.

2.7.2 Measure of cytokine production

$OVA_{(323-339)}$ peptide (ISQAVHAAHAEINEAGR)	Custom synthesis	IAH

$CD4^+$ T cells, purified from lymph nodes , were seeded in a 96-well plate at 1×10^5 cells/well in RF10 growth medium, and incubated with 5×10^5 cells/well syngeneic T-depleted splenocytes (see 2.2.5), in the presence or absence of 5 μM $OVA_{(323-339)}$ peptide. Culture supernatants were collected after 72 h, and assayed for the presence of IL-2, IFN-γ, IL-4, and IL-5 using a Th1/Th2 cytokine CBA kit (see 2.4.1). Cytokine concentration, produced from 1×10^6 cells/mL as described above, was first expressed as pg/mL per 1×10^6 cells, and then data were normalised by calculating, from the percentage of $CD45.2^+$ $CD4^+$, the amount of cytokine secreted by 10^5 OT II cells. Cytokine concentration produced by OVA-specific $CD4^+$ T cells was finally expressed as pg/mL per 10^5 OT II cells.

2.8 Data analysis

Data were represented as mean ± SD, or individual data points and mean value. Analysis of variance was used to evaluate statistical significance, and a probability value of $p<0.05$ was considered significant; calculations were made using the one-way ANOVA test or the ANOVA general linear model, with the Minitab-Release 13.1 software.

Chapter 3

Effects of Toll-like receptor stimuli on adaptive immune responses

3.1 Introduction

The immune system is able to detect pathogens through the recognition of highly conserved structures present in large groups of organisms, such as bacteria, viruses and fungi. Microbe-specific structures have been referred to as pathogen-associated molecular patterns (PAMPs). Best-known PAMPs are peptidoglycan, lipoproteins, flagellin and DNA from bacteria in general, LPS from Gram-negative bacteria, lipoteichoic acids from Gram-positive bacteria, dsRNA and ssRNA from viruses, mannan and glucan from fungi (Krieg, 2002; Akira, 2003; Weber *et al.*, 2003; Masuoka, 2004; Philpott *et al.*, 2004; Kawai *et al.*, 2005a; Miller *et al.*, 2005; Salazar-Gonzalez *et al.*, 2005). Recognition of PAMPs is mediated by receptors referred to as pattern recognition receptors (PRRs). A family of evolutionary conserved PRRs, the Toll-like receptors (TLRs), play an important role in the detection of and response to all classes of pathogens, as mice deficient for various TLRs have increased susceptibility to particular organisms (O'Brien *et al.*, 1980; Woods *et al.*, 1988; Takeuchi *et al.*, 2000; Bellocchio *et al.*, 2004; Tabeta *et al.*, 2004; Zhang *et al.*, 2004). The study of immune responses to purified or synthesised PAMPs in cells transfected with dominant negative TLRs or in mice deficient for individual TLRs has defined some of the specificities of the different TLRs. For instance, zymosan, which is a yeast cell wall fraction (Di Carlo *et al.*, 1958) is recognised by TLR2/TLR6 heterodimers (Ozinsky *et al.*, 2000). Cells from TLR2-deficient mice were found to show impaired responses to peptidoglycan from Gram-positive bacteria (Takeuchi *et al.*, 1999), and it had been previously hypothesised that TLR2 may cooperate with TLR10 to mediate responses to peptidoglycan (Akira, 2003). However, recognition and response to peptidoglycan by TLR2 has since been controversial (Travassos *et al.*, 2004). It is thought that cell wall contaminants in peptidoglycan preparations may have accounted for TLR2 specificity, and that a different set of proteins, Nod1 and Nod2, mediate responses to peptidoglycan

(Philpott *et al.*, 2004). Viral dsRNA and synthetic dsRNA (poly(I:C)) can be recognised by TLR3 (Alexopoulou *et al.*, 2001). Responses to viral ssRNA, synthetic ssRNA and guanosine analogs are mediated by TLR7/TLR8 (Hemmi *et al.*, 2002; Diebold *et al.*, 2004; Heil *et al.*, 2004; Lund *et al.*, 2004). Responses to CpG DNA are mediated by TLR9 (Hemmi *et al.*, 2000).

Activation of adaptive immune responses against pathogens is essential for resolving infections. Recognition of PAMPs by TLRs not only stimulates the production of the costimulatory signals indispensable to the priming of adaptive immune cells, but can also promote specific responses that are tailored to pathogens through the activation of differential cytokine and chemokine gene expression. Indeed, lipopeptide PAM_3CysK_4 (TLR2/TLR1), poly(I:C) (TLR3), LPS from *E. coli* (TLR4), imidazoquinoline R-848 (TLR7) and CpG DNA (TLR9) can induce up-regulation on antigen presenting cells of costimulatory molecules CD80, CD86 and CD40, which are necessary to provide the second signal for T cell differentiation into effector cells (Hemmi *et al.*, 2000; Hemmi *et al.*, 2002; Hoebe *et al.*, 2003b; Lore *et al.*, 2003; Dillon *et al.*, 2004). These PAMPs also stimulate the production of pro-inflammatory (TNF-α, IL-1, IL-6) and immunomodulatory (IL-12, IL-4, IFN-α/β, IFN-γ) cytokines, although different PAMPs may stimulate different classes of responses (Hoshino *et al.*, 1999; Hirschfeld *et al.*, 2001; Agrawal *et al.*, 2003; Yamamoto *et al.*, 2003), and references above].

Hence, by modulating the expression of cytokines and costimulatory molecules, PAMPs play an important role in directing appropriate adaptive immune responses. In addition, many PAMPs have been shown to act as adjuvants on antigen-specific responses. Enhancement of antibody responses against T-dependent antigens by LPS from *E. coli* was described early on (Skidmore *et al.*, 1975; Moreno *et al.*, 1984). Some of the effects of LPS from *E. coli* on antigen-specific $CD4^+$ and $CD8^+$ T cells have been described (Pape *et al.*, 1997; Pulendran *et al.*, 2001; Hoebe *et al.*, 2003b). Poly(I:C), and imidazoquinolines and CpG DNA have also been shown to enhance antigen-specific responses (Mota *et al.*, 1975; Tighe *et al.*, 2000; Vasilakos *et al.*, 2000; Lore *et al.*, 2003; Ahonen *et al.*, 2004). Augmented antigen-specific adaptive responses in response to Mycobacteria products were shown to depend on TLR signalling (Schnare *et al.*, 2001).

$CD8^+$ T cell responses are an important component of adaptive immune responses directed against intracellular pathogens. Priming of $CD8^+$ T cells occurs when $CD8^+$ T cells bind MHC I-peptide complexes on APC, in the presence of licensing signals such as CD40L/CD40 interactions (Guerder *et al.*, 1992; Lanzavecchia, 1998). This model describes the direct priming of $CD8^+$ T cells by APC that present peptides from endogenous antigens. As explained in Chapter 1, activation and differentiation of $CD8^+$ T cells against exogenously acquired antigens, presented on MHC I, can also take place, provided that APC and T cells have received appropriate signals. Priming of antigen-specific effector $CD8^+$ T cells against exogenous antigens is referred to as cross-priming. Some PAMPs, such as poly(I:C) and CpG DNA have been found to enhance cross-priming induced by virus-like particles (Schwarz *et al.*, 2003). Other studies have shown that CpG DNA itself is able to induce cross-priming (Cho *et al.*, 2000; Sparwasser *et al.*, 2000). When the present study was undertaken, it was also shown that cross-priming licensing could be TLR-dependent (Heit *et al.*, 2003).

However, no systematic study of the effect of a broad panel of TLR stimuli on antigen-specific adaptive responses, in particular cross-priming, had been published prior to the studies presented here.

Therefore, the aim of the studies in this chapter was to assess the qualitative effects of a range of TLR agonists on the enhancement of adaptive immune responses against soluble protein antigens *in vivo*. To that end, antigen-specific humoral and $CD8^+$ T cell-mediated responses in the presence of various representative TLR stimuli were characterised in mice.

3.2 Induction of adaptive immune responses by classical TLR agonists

A panel of representative agonists for various TLR was selected (Table 3.1). Doses of zymosan (Ara *et al.*, 2001), peptidoglycan (Tomasic *et al.*, 2000), poly(I:C) (Le Bon *et al.*, 2001), LPS (Ohta *et al.*, 1985; Schulz *et al.*, 2000; Pulendran *et al.*, 2001; Jones, 2004), R-848 (Tomai *et al.*, 2000; Hemmi *et al.*, 2002) and CpG DNA (Schwarz *et al.*, 2003) were chosen in accordance with previous *in vivo* studies, and it was verified that different doses of the same agonist did not have different effects on the enhancement of adaptive immune responses.

Table 3.1. Toll-like receptor agonists used in this study

TLR	TLR2/6	TLR2/?	TLR3	TLR4	TLR7	TLR9
Agonist(s)	Zymosan (*S. cerevisiae*)	Peptidoglycan (*S. aureus*)	Poly(I:C)	LPS (*E. coli*)	R-848	CpG 2216 CpG 1668

3.2.1 Effect of TLR agonists on induction of antigen-specific humoral responses

In order to test the capacity of TLR agonists to elicit humoral responses against a soluble protein antigen, and evaluate the quality of this response, serum titres of antigen-specific antibodies of different IgG isotypes were measured. Chicken gamma globulin (CGG) is a poorly immunogenic protein. It has been used previously to characterise adjuvants (Le Bon *et al.*, 2001; Jones, 2004), and was also chosen here as a model protein antigen. IgG responses were characterised first during a primary response (early on and two months after immunisation), then during a secondary response.

3.2.1.1 Primary response

3.2.1.1.1 Early immunoglobulin G responses

C57Bl/6 mice were injected once intramuscularly (i.m.) with CGG alone, or CGG in combination with different TLR agonists. Titres of CGG-specific IgG1, IgG2a, IgG2b and IgG3 were measured in the serum ten days later, using ELISA (see 2.5.2).

Consistent with the results of Le Bon and colleagues, CGG induced relatively weak IgG responses against itself, consisting mainly of IgG1 (mean titre ± SD: 26,667 ± 19,610) and very little IgG2a (583 ± 719), IgG2b (1,867 ± 998) or IgG3 (317 ± 342). Co-administration of TLR agonists increased CGG-specific IgG responses, each agonist enhancing the production of one or more IgG isotypes (Figure 3.1). Results are expressed as fold increase in antibody endpoint titre compared to titres induced by CGG alone.

Titres of anti-CGG IgG1 were higher in mice immunised with CGG + zymosan, CGG + poly(I:C) or CGG + CpG 2216, compared to IgG1 titres in mice injected with CGG alone (Table 3.2). Peptidoglycan, LPS, R-848 and CpG 1668 had no effect on this response.

All agonists except peptidoglycan were able to increase anti-CGG IgG2a titres, albeit to a varying extent. Potent responses were induced by zymosan, poly(I:C), CpG 2216 and CpG 1668, while LPS induced a moderate but significant increase in levels of CGG-specific IgG2a. R-848 had a weak effect (5.9 ± 3.9-fold increase).

Anti-CGG IgG2b responses were also enhanced significantly. The greatest increases in IgG2b titres were induced by zymosan, poly(I:C) and LPS, to an equal extent, and by CpG 2216. CpG 1668 and R-848 also increased CGG-specific IgG2b levels.

A few TLR agonists had an effect on the generation of anti-CGG IgG3. Poly(I:C), CpG 2216 and CpG 1668 enhanced the response by over 50-fold. Levels of IgG3 were also increased by LPS. R-848 was a weak inducer of anti-CGG IgG3 (5.5 ± 3.6-fold increase), while zymosan and peptidoglycan were both ineffective.

Table 3.2. Enhancement of primary anti-CGG antibody responses by TLR agonists.

	CGG + zymosan	CGG + poly(I:C)	CGG + LPS	CGG + R-848	CGG + CpG 2216	CGG + CpG 1668
IgG1	7.7 ± 0.0 p=0.000	6.4 ± 1.8 p=0.017	< 6.0	< 6.0	7.7 ± 0.0 p=0.000	< 6.0
IgG2a	32.9 ± 15.5 p=0.044	58.5 ± 20.7 p=0.017	7.3 ± 2.6 p=0.036	< 6.0	73.1 ± 20.7 p=0.008	21.9 ± 0.0 p=0.000
IgG2b	91.4 ± 25.9 p=0.008	91.4 ± 25.9 p=0.008	91.4 ± 25.9 p=0.008	18.3 ± 6.5 p=0.020	128 ± 68.4	36.6 ± 12.9 p=0.018
IgG3	134.7 ± 133.4	94.3 ± 50.4 p=0.022	188.6 ± 100.8	< 6.0	134.7 ± 38.1 p=0.008	53.9 ± 19.1 p=0.017

p, statistical significance determined by one-way ANOVA test (titres induced by CGG + agonist *versus* titres induced by CGG alone).
The table shows fold increases in anti-CGG antibody endpoint titres at day 10, compared to titres in mice injected with CGG alone, in mice immunised once at day 0 with CGG and TLR agonist (mean ± SD, n=3). Only agonists that induced at least a seven-fold enhancement of fold increase in anti-CGG antibody endpoint titres are included in the above table.

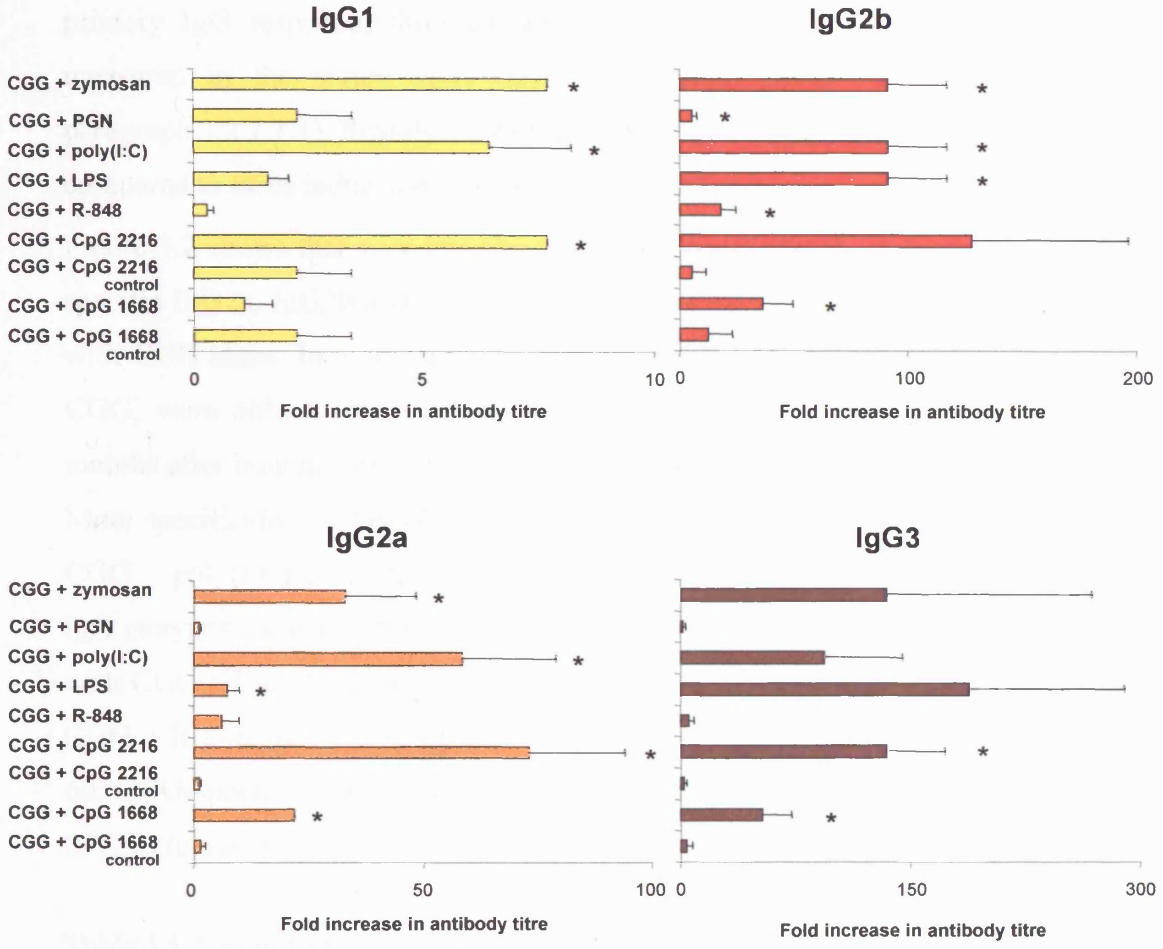

Figure 3.1. The effect of TLR agonists on primary antigen-specific antibody responses. C57Bl/6 mice were injected i.m. with 100 μg chicken γ-globulin (CGG) alone or CGG in combination with zymosan (5 mg), peptidoglycan from *S. aureus* (PGN) (200 μg), poly(I:C) (100 μg), LPS from *E. coli* (10 μg), R-848 (200 μg), CpG 2216 (20 nmol), CpG 2216 control (20 nmol), CpG 1668 (20 nmol) or CpG 1668 control (20 nmol). Ten days after immunisation, serum samples were collected and titres of CGG-specific IgG1, IgG2b, IgG2a and IgG3 were measured using ELISA. Results are expressed as fold-increase in CGG-specific antibody titre compared with titres in mice immunised with CGG alone. Data are represented by mean ± SD for three mice per group. * $p<0.05$ versus CGG immunisation group, by one-way ANOVA test.

3.2.1.1.2 Long-term immunoglobulin G responses

In order to test whether TLR agonists could generate a long-lasting CGG-specific primary IgG response, titres of anti-CGG IgG1, IgG2a, IgG2b and IgG3 were measured in the serum sixty days after immunisation (protocol described in paragraph 3.2.1.1.1). Results are expressed as fold increase in antibody endpoint titre compared to titres induced by CGG injected alone.

Figure 3.2 shows that very low titres of CGG-specific IgG1, and virtually no CGG-specific IgG2a, IgG2b and IgG3 were present at day 60 in mice previously injected with CGG alone. In contrast, most agonists, co-administered sixty days earlier with CGG, were able to elicit anti-CGG IgG responses that were still stronger, two months after immunisation, than responses in mice injected with CGG alone.

More specifically, at day 60, in mice previously immunised with CGG + zymosan, CGG + poly(I:C) or CGG + CpG DNA, CGG-specific antibodies of three or more IgG isotypes were still present and at higher titres than in mice previously injected with CGG alone (Table 3.3). Responses enhanced by a previous immunisation with CGG + R-848 were rather restricted to anti-CGG IgG2b and IgG3 isotypes. At day 60, CGG-specific antibody titres in mice immunised with CGG + PGN or CGG + LPS were not much higher than titres in mice injected with CGG alone.

Table 3.3. Enhancement of long-term anti-CGG antibody responses by TLR agonists.

	CGG + zymosan	CGG + poly(I:C)	CGG + R-848	CGG + CpG 2216	CGG + CpG 1668
IgG1	49.2 ± 13.9 p=0.008	16.0 ± 10.6	8.6 ± 4.6	12.3 ± 3.5 p=0.011	< 6.0
IgG2a	12.8 ± 4.5 p=0.023	64.0 ± 18.1 p=0.008	< 6.0	89.6 ± 47.9	22.4 ± 12.0
IgG2b	332.8 ± 220.2	153.6 ± 0.0 p=0.000	102.4 ± 36.2 p=0.017	281.6 ± 237.4	89.6 ± 47.9
IgG3	< 6.0	10.7 ± 3.8 p=0.022	224.0 ± 207.4	86.7 ± 58.5	224.0 ± 207.4

p, statistical significance determined by one-way ANOVA test (titres induced by CGG + agonist *versus* titres induced by CGG alone).

The table shows fold increases in anti-CGG antibody endpoint titres at day 60, compared to titres in mice injected with CGG alone, in mice immunised once at day 0 with CGG and TLR agonist (mean ± SD, n=3). Only agonists that induced at least a seven-fold enhancement of fold increase in anti-CGG antibody endpoint titres are included in the above table

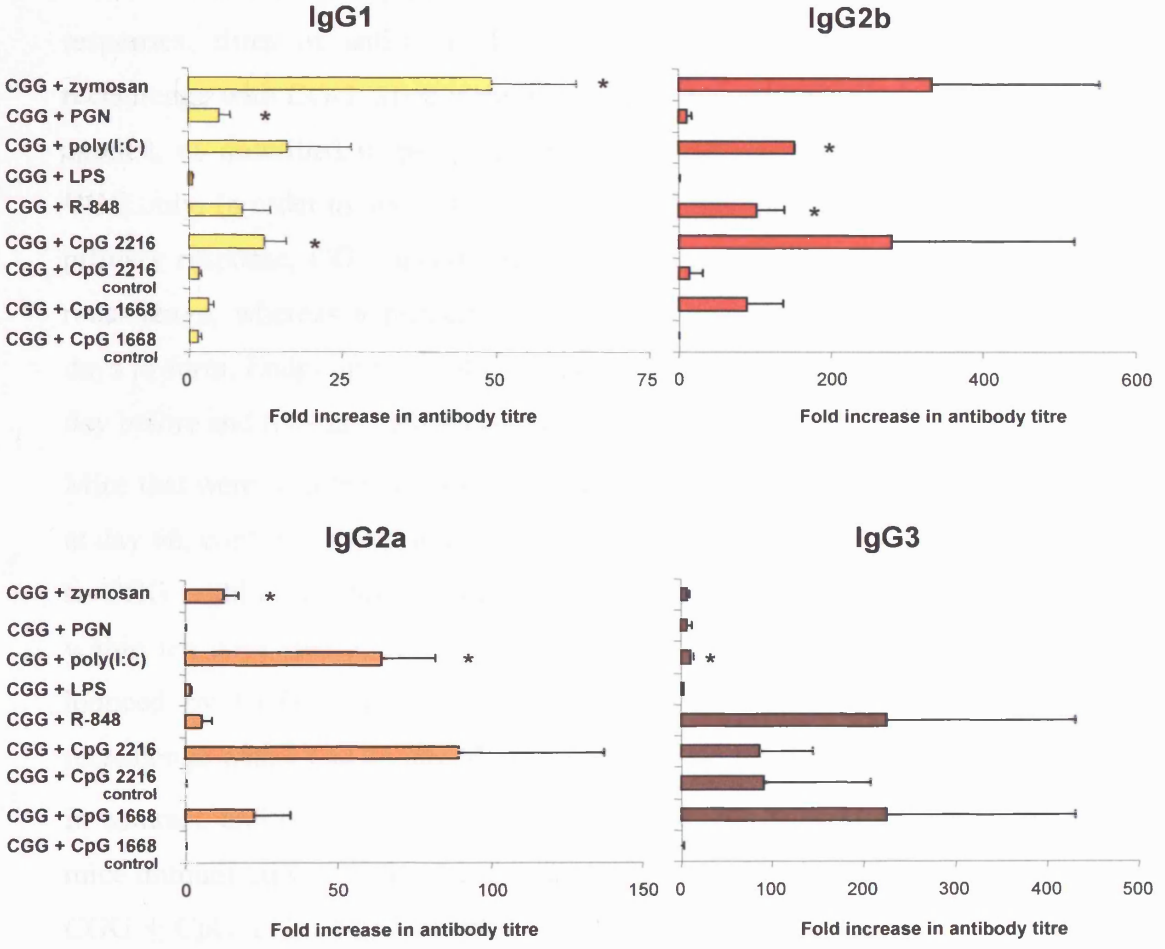

Figure 3.2. The effect of TLR agonists on long-term antigen-specific antibody responses. C57Bl/6 mice were injected i.m. with 100 μg chicken γ-globulin (CGG) alone or CGG in combination with zymosan (5 mg), peptidoglycan from *S. aureus* (PGN) (200 μg), poly(I:C) (100 μg), LPS from *E. coli* (10 μg), R-848 (200 μg), CpG 2216 (20 nmol), CpG 2216 control (20 nmol), CpG 1668 (20 nmol) or CpG 1668 control (20 nmol). Sixty days after immunisation, serum samples were collected and titres of CGG-specific IgG1, IgG2b, IgG2a and IgG3 were measured using ELISA. Results are expressed as fold-increase in CGG-specific antibody titre compared with titres in mice immunised with CGG alone. Data are represented by mean ± SD for three mice per group. * $p<0.05$ versus CGG immunisation group, by one-way ANOVA test.

3.2.1.2 Secondary response

In order to test the ability of TLR agonists to generate memory antigen-specific IgG responses, titres of anti-CGG IgG isotypes were measured in the serum after rechallenge with CGG. Mice were immunised once with CGG alone or with CGG + agonist, as described in paragraph 3.2.1.1.1, and rechallenged 61 days later with CGG only. In order to assess the mounting of a memory response, rather than a new primary response, CGG-specific titres were measured in the serum five days after rechallenge, whereas a primary IgG response would necessitate more than seven days to form. Endpoint titres of CGG-specific IgG1, IgG2a, IgG2b and IgG3, on the day before and five days after rechallenge, are shown in Table 3.4 and Figure 3.3.

Mice that were injected only once with CGG, at day 61, had no anti-CGG antibodies at day 66, confirming that mice were not able to mount a primary antibody response to CGG within five days, as opposed to the low primary response that developed within ten days (see 3.2.1.1.1). CGG-specific IgG1, IgG2a, IgG2b and IgG3 titres induced by CGG, injected alone at day 0 first, increased insignificantly after rechallenge with CGG on day 61.

In contrast, anti-CGG IgG1 titres increased efficiently after antigenic rechallenge in mice immunised with CGG + zymosan, CGG + peptidoglycan, CGG + poly(I:C) and CGG + CpG 2216. Mice initially immunised with CGG + LPS, CGG + R-848 or CGG + CpG 1668 were also able to mount a secondary response.

All agonists except peptidoglycan elicited anti-CGG IgG2a responses that could be significantly recalled upon rechallenge. In particular, zymosan, poly(I:C) and CpG 1668 were very potent inducers of IgG2a memory responses. In mice immunised with CGG + R-848, CGG + LPS or CGG + CpG 2216, anti-CGG IgG2a titres after rechallenge were over 30 times greater than before rechallenge.

Secondary anti CGG-IgG2b responses were most significant in mice immunised with CGG + zymosan and CGG + CpG 2216. Recall responses also developed, though to a lesser extent, in mice immunised with CGG + peptidoglycan, CGG + LPS or CGG + CpG 1668. Mice immunised with CGG + R-848 or CGG + poly(I:C) were not able to mount a proper secondary anti-CGG IgG2b response.

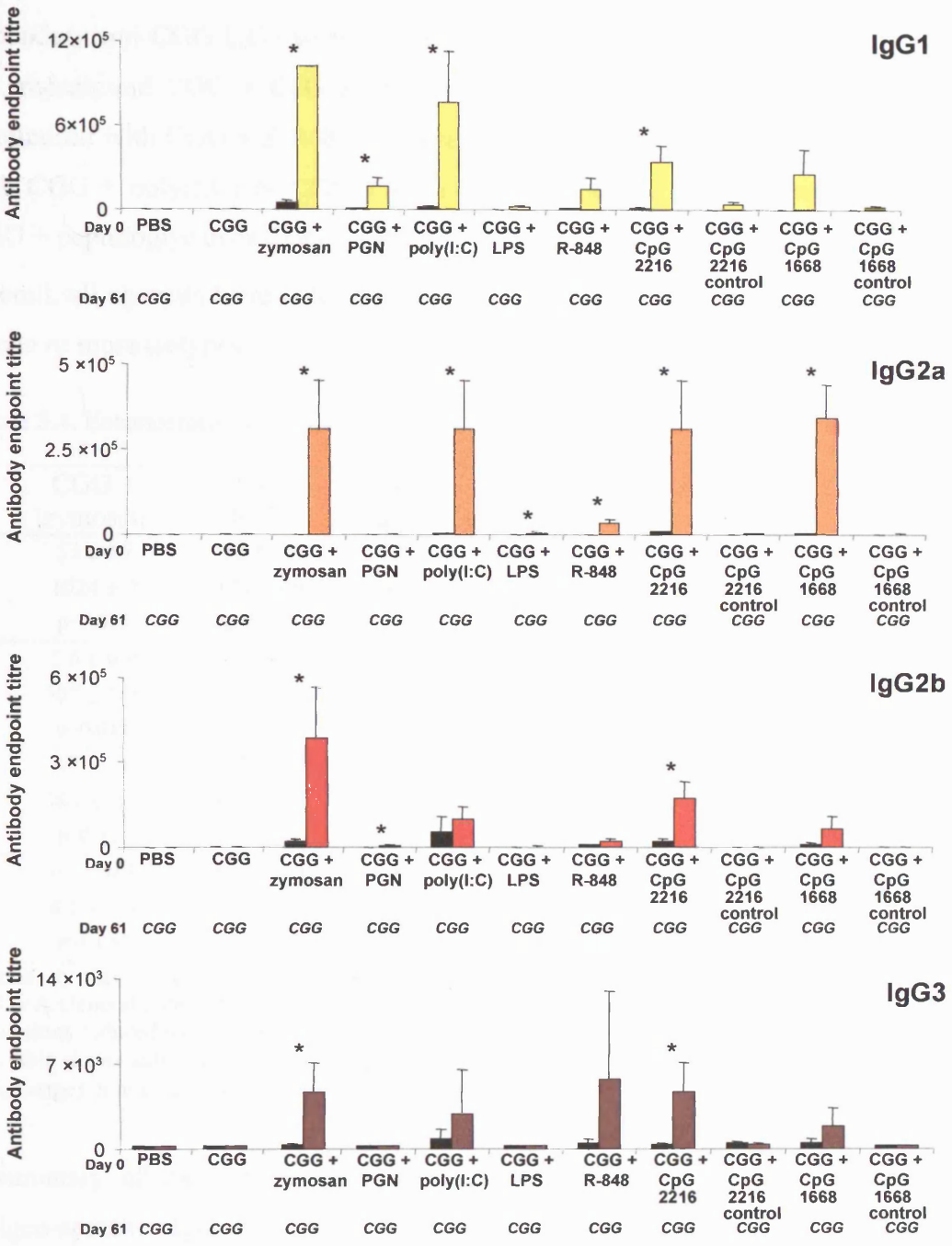

Figure 3.3. The effect of TLR agonists on secondary antigen-specific antibody responses. C57Bl/6 mice were injected i.m. with PBS or 100 μg chicken γ-globulin (CGG) alone or CGG in combination with zymosan (5 mg), peptidoglycan from *S. aureus* (PGN) (200 μg), poly(I:C) (100 μg), LPS from *E. coli* (10 μg), R-848 (200 μg), CpG 2216 (20 nmol), CpG 2216 control (20 nmol), CpG 1668 (20 nmol) or CpG 1668 control (20 nmol). Sixty-one days after immunisation, mice were rechallenged i.p. with 100 μg CGG. Six days later, serum samples were collected and titres of CGG-specific IgG1, IgG2a, IgG2b and IgG3 before rechallenge (■) and after rechallenge (▯▯▯▯) were measured using ELISA. Results are expressed as CGG-specific antibody endpoint titre. Data are represented by mean ± SD for three mice per group. * $p<0.05$ versus CGG immunisation groups (before and after challenge), by ANOVA General Linear Model test.

Secondary anti-CGG IgG3 were also most significant in mice immunised with CGG + zymosan and CGG + CpG 2216. Titres increased more than ten times in mice immunised with CGG + R-848. Recall responses were moderate in mice immunised with CGG + poly(I:C) or CGG + CpG 1668, but absent in mice immunised with CGG + peptidoglycan or CGG + LPS.

Overall, all agonists were able to induce significant recall anti-CGG IgG responses, of one or more isotypes.

Table 3.4. Enhancement of memory anti-CGG antibody responses by TLR agonists.

		CGG + zymosan	CGG + PGN	CGG + poly(I:C)	CGG + LPS	CGG + R-848	CGG + CpG 2216	CGG + CpG 1668
IgG1	I	53 ± 15	53 ± 19	17 ± 11	0.6 ± 0.3	9.3 ± 5.0	13 ± 4	3.3 ± 0.9
	II	1024 ± 0	171 ± 60	768 ± 362	19 ± 10	149 ± 80	341 ± 121	256 ± 18
		p=0.000	p=0.005	p=0.019			p=0.005	
IgG2a	I	1.6 ± 0.0	0.1 ± 0.0	4.3 ± 1.5	0.1 ± 0.0	0.7 ± 0.2	6.4 ± 0.0	2.4 ± 1.1
	II	307 ± 145	1.7 ± 1.1	307 ± 145	5.3 ± 1.5	34 ± 12	307 ± 145	341 ± 97
		p=0.018		p=0.018	p=0.001	p=0.004	p=0.018	p=0.001
IgG2b	I	17 ± 11	1.2 ± 0.6	55 ± 53	0 ± 0	8 ± 0	19 ± 10	8.7 ± 5.7
	II	384 ± 181	6.7 ± 1.9	96 ± 45	2.3 ± 1.2	19 ± 10	171 ± 60	64 ± 45
		p=0.021					p=0.008	
IgG3	I	0.3 ± 0.1	0.2 ± 0.0	0.8 ± 0.8	0.2 ± 0.0	0.5 ± 0.3	0.3 ± 0.1	0.5 ± 0.3
	II	4.7 ± 2.3	0.2 ± 0.0	2.8 ± 3.6	0.2 ± 0.0	5.8 ± 7.3	4.7 ± 2.5	1.8 ± 1.5
		p=0.037					p=0.04	

I, antibody titres at day 60. II, antibody titres at day 66. p, statistical significance determined by ANOVA General Linear Model test (titres induced by CGG + agonist at day 66 compared to day 60 *versus* titres induced by CGG alone at day 66 compared to day 60).
The table shows anti-CGG antibody endpoint titres at day 60 (before rechallenge) and at day 66 (after rechallenge) in mice immunised once at day 0 with CGG and TLR agonist (mean $\times 10^3 \pm$ SD, n=3).

In summary, all the TLR agonists tested were able to induce primary and secondary antigen-specific IgG responses, with both responses characterised by isotype switching. Thus, all have adjuvant properties in the induction of humoral responses. Overall, the addition of zymosan, poly(I:C) or CpG DNA to a soluble protein antigen generated or increased primary and memory IgG responses of all isotypes, while LPS, R-848 and peptidoglycan had a more restricted effect.

In addition to studying the effects of TLR agonists on the enhancement of adaptive humoral responses, a major aim of this work was to investigate the effect of TLR agonists on $CD8^+$ T cell responses, more precisely their potential to induce cross-priming.

3.2.2 Effect of TLR agonists on induction of antigen-specific $CD8^+$ T cell responses

As described in Chapter 1.2.2.3.2, cross-priming refers to the generation of functional $CD8^+$ T cell responses against an exogenous antigen. The ability of TLR agonists to induce cross-priming against a soluble antigen was assessed here. Ovalbumin (OVA) was chosen as a model protein antigen to test the capacity of TLR agonists to stimulate antigen-specific CD8 T^+ cell responses. OVA is a poorly immunogenic protein, which constitutes an efficient tool to follow antigen-specific $CD8^+$ T cell as well as $CD4^+$ T cell responses. Indeed, dominant epitopes have been described for mouse MHC I (OVA $_{257-264}$) and MHC II (OVA $_{323-339}$) and transgenic mice, with TCR specific for each MHC-peptide complex, have been engineered. OVA was thus chosen as a model soluble protein antigen, to study antigen-specific $CD8^+$ and $CD4^+$ responses

C57Bl/6 mice, which have a $H\text{-}2^b$ haplotype, received a single injection i.m. with OVA either alone or in combination with a TLR agonist. To first assess whether TLR agonists were able to induce expansion of OVA-specific $CD8^+$ T cells, the frequency of $CD8^+$ T cell bearing SIINFEKL-specific TCR was measured in spleen eight days after immunisation, using $H\text{-}2K^b$-SIINFEKL tetramers and FACS analysis (see 2.6.1). Representative FACS analysis dot plots are shown in Figure 3.4A. No tetramer-positive (Tet^+) cells could be detected in naïve mice or mice injected with OVA (Figure 3.4B). Similarly, zymosan, peptidoglycan, LPS or R-848 could not induce any detectable OVA-specific $CD8^+$ T cells population. In contrast, co-administration of poly(I:C) or CpG DNA (CpG 2216) with OVA induced expansion of SIINFEKL-specific $CD8^+$ T cells: 1.2 % Tet^+ ± 0.4 and 0.6% ± 0.1, respectively.

As a first approach to assess the generation of functional OVA-specific $CD8^+$ T cells, SIINFEKL-specific $CD8^+$ T cells able to produce IFN-γ were enumerated *ex vivo* after restimulation *in vitro*, using IFN-γ ELISPOT assay (see 2.6.2). Spots were counted to evaluate the frequency (*f*) of OVA-specific $CD8^+$ T cells producing IFN-γ; *f* was expressed as the number of IFN-γ spots per 10^6 $CD8^+$ T cells (Figure 3.4C).

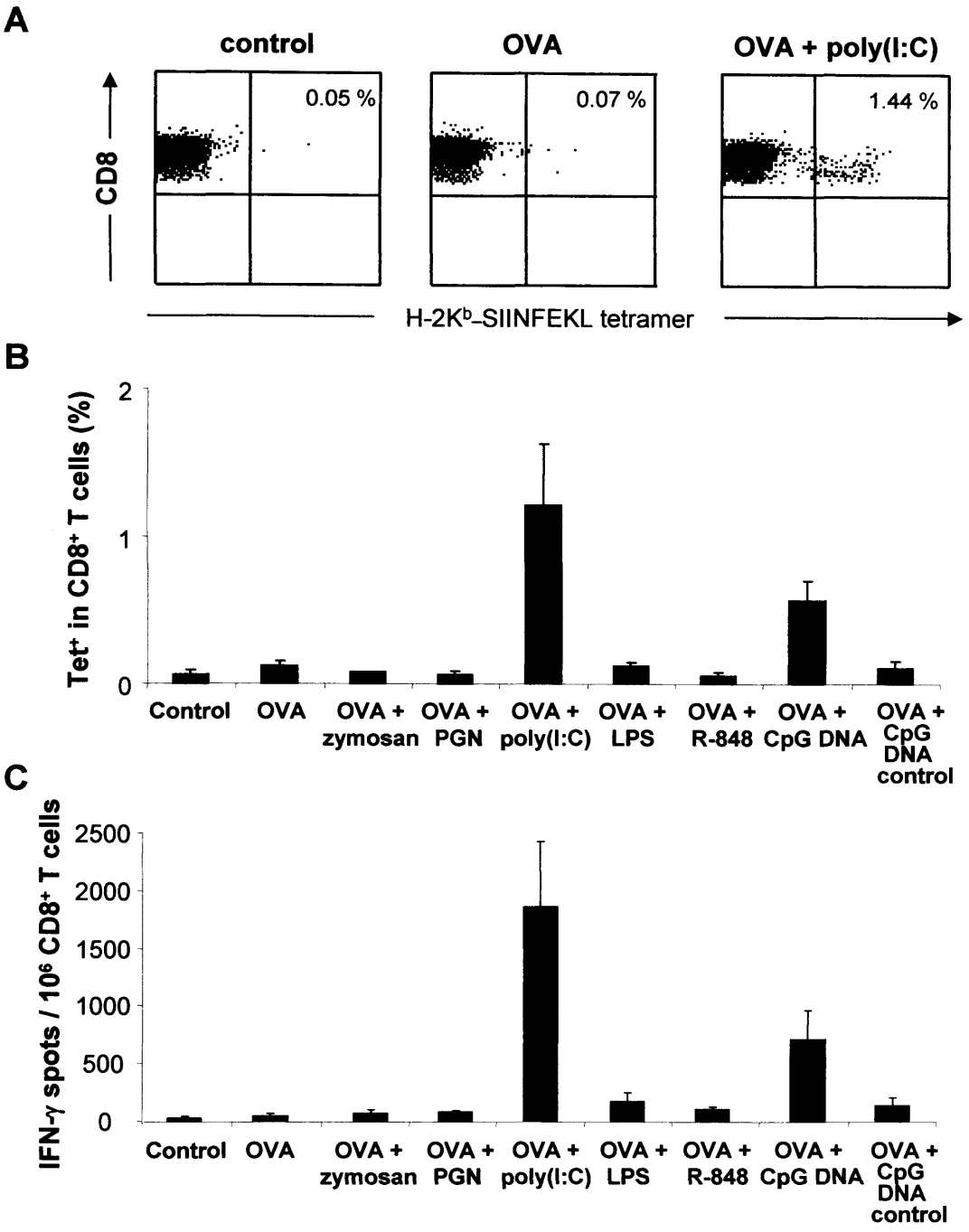

Figure 3.4. TLR 3 and TLR 9 agonists induce functional cross-priming against OVA. C57Bl/6 mice were injected i.m. with 500 μg ovalbumin (OVA) alone or OVA in combination with zymosan (500 μg), peptidoglycan from *S. aureus* (PGN) (100 μg), poly(I:C) (100 μg), LPS from *E. coli* (10 μg), R-848 (50 nmol), CpG 2216 (20 nmol) or CpG 2216 control (20 nmol). (A,B) Eight days after immunisation, SIINFEKL-specific CD8$^+$ T cells in spleens were quantified using K^b-SIINFEKL tetramer staining. (A) Representative FACS dot plots of tetramer staining. The value in the right corner of each panel represents the percentage of tetramer-positive (Tet$^+$) cells among CD8$^+$ T cells. (B) Percentage of Tet$^+$ cells gated on CD8$^+$ T cells. Data are represented by mean ± SD for three mice per group. (C) Eight days after immunisation, splenic CD8$^+$ T cells were assessed for SIINFEKL-specific IFN-γ secretion by ELISPOT assay. Results are expressed as the number of spots per 10^6 CD8$^+$ T cells. Data are represented by mean ± SD for three mice per group.

No spots could be detected after stimulation in the absence of peptide. An increase in the number of spots, in comparison to naïve mice (2 spots ± 1, *f*=27 ± 12) or mice injected with OVA alone (3 spots ± 2, *f*=42 ± 25), was only detectable from mice immunised with OVA + poly(I:C) (124 spots ± 29, *f*=1861 ± 564) or OVA + CpG 2216 (47 spots ± 18, *f*=703 ± 255).

Results from tetramer staining and ELISPOT assay showed that immunisation with OVA in combination with poly(I:C) or CpG DNA induced OVA-specific $CD8^+$ T cell responses, and that some of the antigen-specific $CD8^+$ T cells generated had differentiated into effector cells producing IFN-γ.

To characterise further the functional properties of OVA-specific $CD8^+$ T cells generated in the presence of TLR stimuli, the cytotoxic potential of these cells was assessed.

The principal mechanism used by cytotoxic $CD8^+$ T cells to induce apoptosis of target cells is the secretion of lytic granules into the immunological synapse. Since granzymes, cell-death-inducing enzymes, are essential components of the granules, it was of interest to measure their expression in antigen-specific $CD8^+$ T cells generated in the presence TLR stimuli. However, reagents to detect any of the murine granzymes species were not commercially available, and only the anti-human granzyme B antibody provided by Caltag Laboratories (Burlingame, CA, USA) was known to cross-react with mouse granzyme B. Expression of granzyme B by OVA-specific $CD8^+$ T cells was thus examined by intracellular staining (see 2.6.1) and FACS analysis (Figure 3.5). Representative FACS analysis histograms are shown in Figure 3.5A. The shift in fluorescence intensity illustrated the expression of granzyme B by OVA-specific $CD8^+$ T cells, in mice immunised with OVA + poly(I:C) or OVA + CpG DNA. Distribution of granzyme B expression, in the $CD8^+/Tet^+$ cell population, was also examined (Figure 3.5B). Results showed that 20.3% ± 3.2 of OVA-specific $CD8^+$ T cells expressed granzyme B after immunisation with OVA + poly(I:C), and 35.1% ± 6.0 after immunisation with OVA + CpG DNA.

Expression of granzyme B is another indication that antigen-specific $CD8^+$ T cells, primed in the presence of poly(I:C) or CpG DNA, have differentiated into effector cells that are potentially cytolytic.

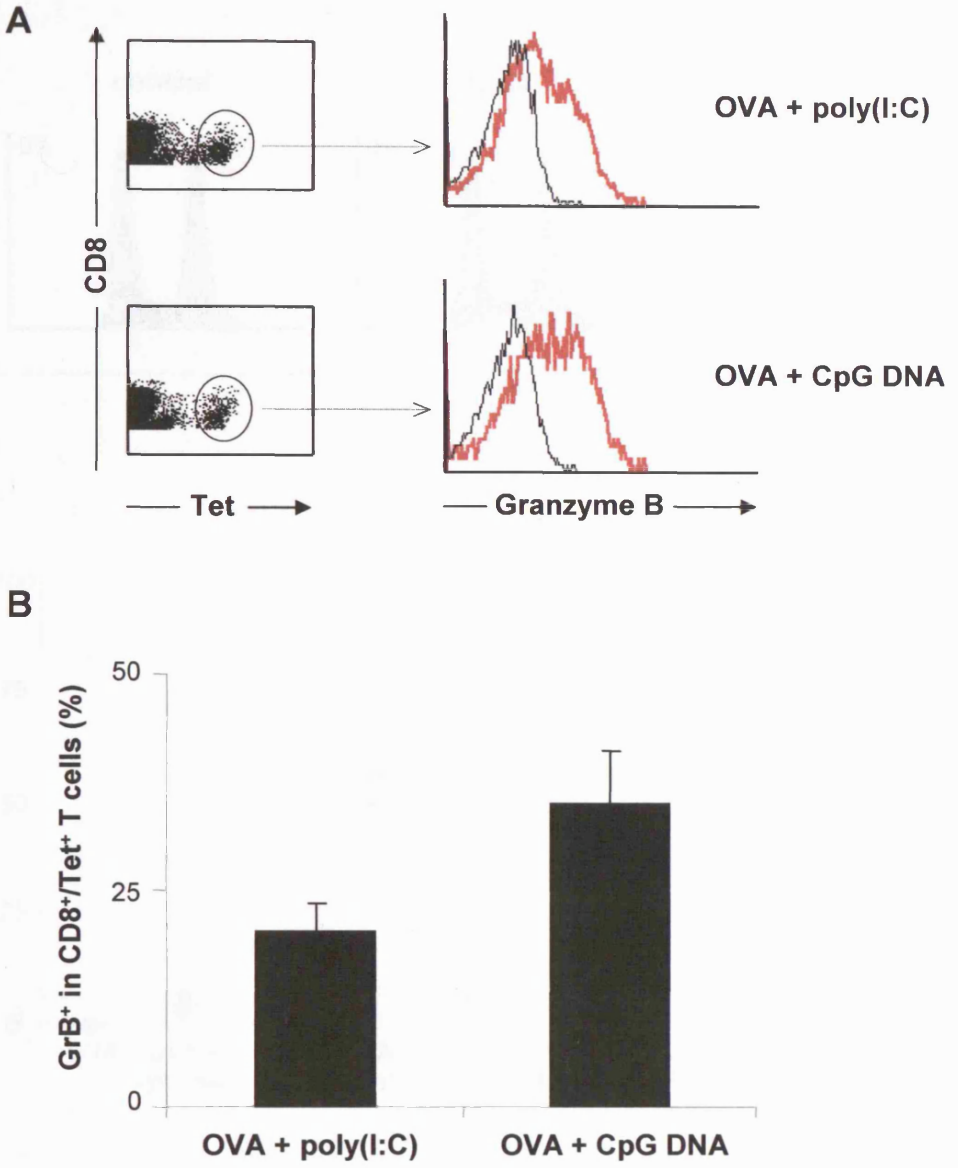

Figure 3.5. TLR 3 and TLR 9 agonists induce the production of cytolytic enzymes in antigen-specific $CD8^+$ T cells. C57Bl/6 mice were injected i.m. with OVA (500 µg) in combination with poly(I:C) (100 µg) or CpG 2216 (20 nmol). Eight days after immunisation, splenic $CD8^+$ T cells were stained with K^b-SIINFEKL tetramer and for intracellular granzyme B. (A) Representative FACS histograms of splenocytes from a mouse immunised with OVA + poly(I:C) or OVA + CpG 2216. Splenocytes, enriched in $CD8^+$ T cells, were stained with anti-granzyme B antibody (—) or isotype control (—). Events were gated on $CD8^+$ tetramer-positive (Tet^+) T cells. (B) Results are expressed as percentage of granzyme B-positive cells (GrB^+), with events gated on $CD8^+$ Tet^+ cells. Data are represented by mean ± SD for three mice per group.

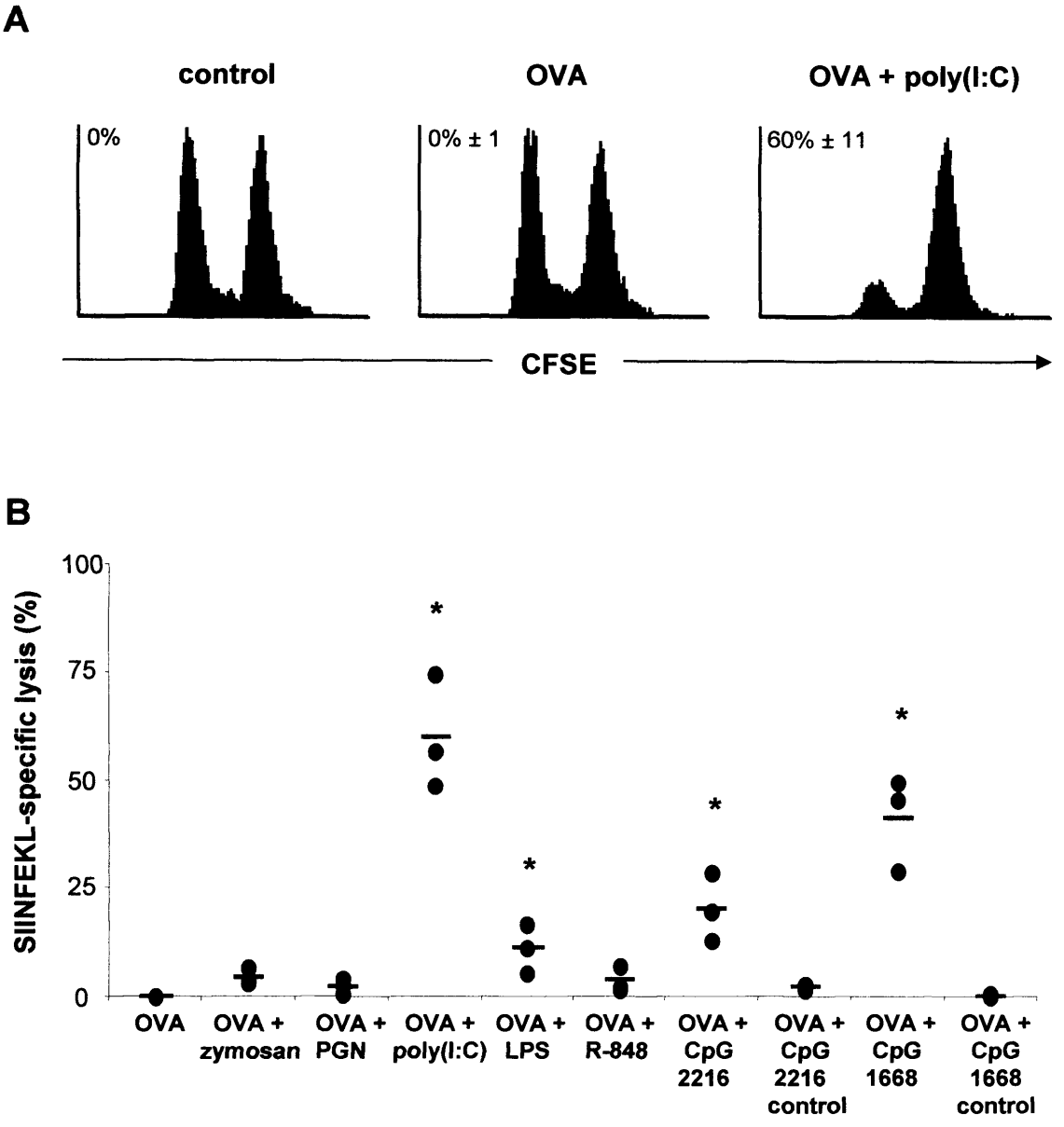

Figure 3.6. TLR 3, TLR 4 and TLR 9 agonists induce antigen-specific cytotoxicity *in vivo*. C57Bl/6 mice were injected i.m. with 500 μg OVA alone or OVA in combination with zymosan (5 mg), peptidoglycan from *S. aureus* (PGN) (180 μg), poly(I:C) (100 μg), LPS from *E. coli* (10 μg), R-848 (260 nmol), CpG 2216 (20 nmol), CpG 2216 control (20 nmol), CpG 1668 (20nmol) or CpG 1668 control (20 noml). Nine days after immunisation, SIINFEKL-specific $CD8^+$ T cell cytotoxic activity was determined by *in vivo* CTL assay: naïve splenocytes were pulsed with SIINFEKL, labelled with $CFSE^{low}$ and co-injected i.v., in a 1:1 ratio, with $CFSE^{high}$ control splenocytes, into immunised and control mice. (A) Representative FACS histograms of CFSE-positive cells recovered from the spleen of immunised and control mice, 18 h after i.v. injection. The value in the left corner of each panel represents the percentage of SIINFEKL-specific cytotoxicity. (B) Results are expressed as percentage of SIINFEKL-specific lysis for three individual mice per group; horizontal lines represent the mean percentage of lysis for each group. * $p<0.05$ versus control by one-way ANOVA test.

To specifically test the cytotoxic function of OVA-specific $CD8^+$ T cells, CTL assays were performed *in vivo* (see 2.6.3). Representative FACS analysis histograms, showing loss of SIINFEKL-pulsed targets cells in a mouse immunised with OVA + poly(I:C), compared to a control mouse and as opposed to a mouse injected with OVA alone, are presented in Figure 3.6A. Data from the *in vivo* CTL assay are presented as percentage of SIINFEKL-specific lysis (see 2.6.3.2) (Figure 3.6B). OVA injected on its own did not generate OVA–specific CTL. Only four agonists were able to. In mice immunised with OVA and LPS, 11.1% ± 4.6 ($p=0.035$) of targets were killed. That percentage was 59.9 ± 10.7 ($p=0.013$) in mice immunised with OVA + poly(I:C), 20.5 ± 6.5 ($p=0.002$) with OVA + CpG 2216, and 41.3 ± 8.8 ($p=0.003$) with OVA + CpG 1668. No lysis, or less than an average of 5% lysis occurred in mice immunised with OVA and the other agonists.

In summary, of all TLR agonists tested, only poly(I:C), LPS and CpG DNAs were able to induce functional antigen-specific $CD8^+$ T cell responses.

3.3 Induction of adaptive immune responses by non-classical lipopolysaccharides

Lipopolysaccharides (LPSs) are glycolipids synthesised by Gram-negative bacteria and integrated in the bacteria outer membrane. Although the precise chemical structure of LPSs varies among bacteria species and strains, LPSs generally contain the following three major components: lipid A, which is the endotoxin moiety of LPS (Galanos *et al.*, 1985), the core polysaccharide, and the polysaccharide side chains, also called O-polysaccharide or O-antigen. Lipid A is a disaccharide that carries negatively charged phosphate groups and acylated fatty acids. The exact nature of the disaccharide, the degree of phosphorylation and number/location of negative charges, and the structure, position and number of hydrophobic acyl side-chains contribute to defining the shape of lipid A (Rietschel *et al.*, 1994). For example, lipid A with six and possibly seven acyl chains, such as lipid A from *E. coli* or *Klebsiella pneumoniae*, form a conical structure, while lipid A with five acyl chains, such as from *P. gingivalis*, form a predominantly cylindrical structure (Schromm *et al.*, 1998; Schromm *et al.*, 2000). Importantly, it is thought that immunological properties of LPS may depend on lipid A conformation (Brandenburg

et al., 1993; Schromm *et al.*, 1998; Schromm *et al.*, 2000; Netea *et al.*, 2002; Stover *et al.*, 2004).

The identification of endotoxin-tolerant C3H/HeJ and C57Bl/10ScCr mouse strains (Sultzer, 1968; Watson *et al.*, 1974; Coutinho *et al.*, 1977; Hoffmann *et al.*, 1977; Michalek *et al.*, 1980) and characterisation of a single gene (*Lps*) mutation encouraged the search for the endotoxin receptor(s) (Watson *et al.*, 1978). When the innate immune receptor TLR4 was described (Rock *et al.*, 1998), and assigned to a chromosomal region that corresponded to the location of *Lps*, it was postulated that TLR4 was the receptor for endotoxin/LPS. After cloning of the mouse *tlr4* gene, the exact mutation responsible for the phenotype of C3H/Hej and C57Bl/10ScCr mice was characterised (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999), and the generation and study of TLR4-deficient mice confirmed that immunological responses to endotoxin depended on TLR4 (Hoshino *et al.*, 1999).

TLR4, within the CD14-TLR4-MD-2 complex, is generally considered as the receptor for LPS. The majority of studies investigating the role of TLR4 in recognition of and signalling to endotoxin/LPS have used LPS from *E. coli* or *Salmonella* spp. as prototypic LPSs. However, LPSs from non-enterobacterial organisms, such as LPSs from periodontal bacteria *P. gingivalis* and *Prevotella intermedia* (Kirikae *et al.*, 1999; Hirschfeld *et al.*, 2001; Pulendran *et al.*, 2001), or LPS from atypical spirochete *Leptospira interrogans* (Werts *et al.*, 2001) have been shown to activate immune responses independently of TLR4. *P. gingivalis* lipid A was shown to activate immune cells from endotoxin-tolerant mice (Ogawa *et al.*, 1996; Tanamoto *et al.*, 1997) and it has been hypothesised that the lipid A structure/conformation may be responsible for the fact that LPS from *P. gingivalis* and other TLR4-independent LPSs have a different agonist-receptor specificity (Werts *et al.*, 2001; Erridge *et al.*, 2004).

Neisseria meningitidis synthesise and transport to their outer membrane a glycolipid called lipooligosaccharide (LOS). LOS is analogous to LPS as it shares similar lipid A structures and core polysaccharide, although LOS lacks O-antigen units (Preston *et al.*, 1996). *N. meningitidis* lipid A is hexa-acylated, and its LOS was shown to induce cytokines and chemokines production through CD14/TLR4 signalling pathways (Mirlashari *et al.*, 2003; Moller *et al.*, 2003; Zughaier *et al.*, 2004). LOS from *N.*

meningitidis is often put into the same category as LPS. While still recognising that LOS presents singularities, LOS will be referred to as LPS in the work reported here.

While the effects of various LPS structures on stimulation of innate inflammatory responses have been investigated (Hirschfeld *et al.*, 2001; Werts *et al.*, 2001; Zughaier *et al.*, 2005), little has been reported on the effect of glycolipids with different conformation on adaptive immune responses (Pulendran *et al.*, 2001).

The aim of the studies presented in this section was to determine the capacity of LPSs from different bacteria species to induce functional cross-priming against a soluble protein antigen. LPSs included in this study were from *E. coli*, from *K. pneumoniae*, from *N. meningitidis* and from *P. gingivalis*.

3.3.1 Preparation of lipopolysaccharides

The LPSs prepared are dispersable in aqueous solvents. They could therefore be extracted from the bacterial cell wall using hot aqueous phenol (Westphal *et al.*, 1965) (see 2.3.3). LPS separate into the aqueous phase, while most protein (Morrison *et al.*, 1976) and nucleic acids (Sambrook *et al.*, 1989b) separate into the phenol phase. Since it has been reported that protein contaminants can co-purify with lipid A and/or LPS, and that these endotoxin proteins may have immune activity (Hirschfeld *et al.*, 2000), extracted LPSs were further purified using gel filtration chromatography, with a detergent-containing buffer (Manthey *et al.*, 1994a; Manthey *et al.*, 1994b; Andersen *et al.*, 2002). Sodium deoxycholate in the chromatography running buffer helps dissociating LPS micelles and separate potential endotoxin-associated proteins.

A representative gel filtration elution profile and SDS-PAGE migration pattern are illustrated in Figure 3.7. Chromatograms, obtained from monitoring the absorbance (OD) and refractory index (RI) (Figure 3.7A), show that components with a high OD (above 1.00 AU) and low RI (under 5,000 MV) eluted from the column between 135 min and 150 min after injection. Components with a high RI (above 40,000 MV) but with hardly any OD (less than 0.005) eluted between 230 min and 250 min after injection. Elution fractions with the highest RI contained LPS, while proteins and nucleic acids, carried over from the phenol-extraction procedure, had a high OD but very low RI. More importantly, they eluted 100 min earlier than LPS.

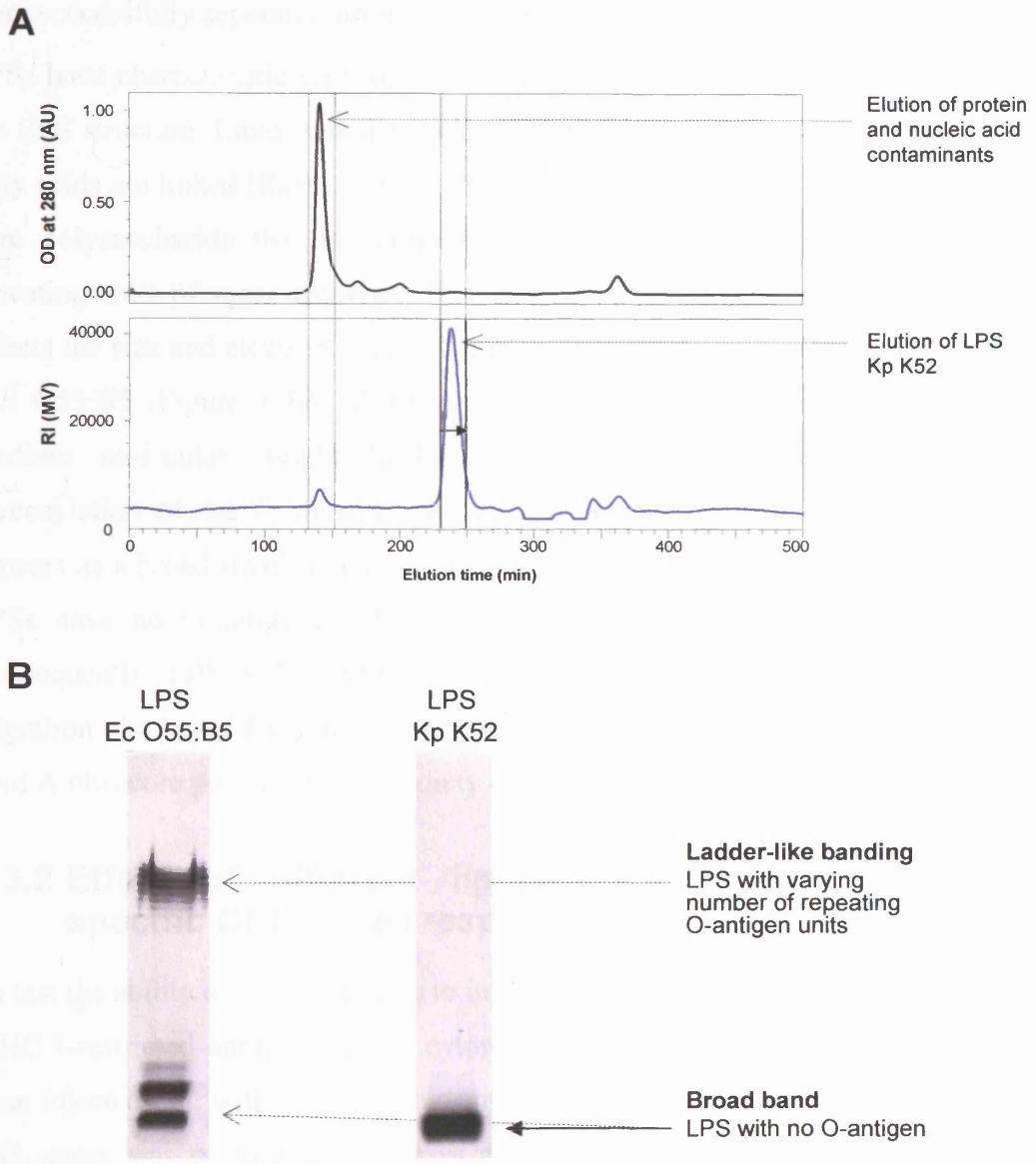

Figure 3.7. Purification and characterisation of lipopolysaccharides: representative chromatography and electrophoresis profiles. After hot-phenol extraction from bacteria, LPSs were purified by gel filtration in sodium deoxycholate dissociating buffer, and characterised by SDS-PAGE. (A) HPLC elution profile of LPS from *K. pneumoniae* K52 (Kp K52), using a Superdex 75 column. The upper panel represents absorbance at 280 nm and the lower panel shows the refractive index (RI). The arrow (⊢▸) indicates the range of elution fractions that were collected and pooled. (B) Electrophoretic mobility in 12% SDS-polyacrylamide gel of HPLC-purified LPS from *E. coli* O55:B5 (Ec O55:B5) and from *K. pneumoniae* K52 (Kp K52), as visualized by silver staining.

Thus, the chromatograms described above show that major known contaminants were successfully separated from the LPS fraction.

LPSs have characteristic electrophoresis migration patterns, which are in relation to the LPS structure. Lipid A is the lipid fraction of LPS; it is a disaccharide to which fatty acids are linked (Raetz, 1990). The polysaccharide fraction of LPS consists of a core polysaccharide that is connected to the O-polysaccharide, which contains repeating units of sugar sequences that may be branched. The size of the O-antigen affects the size and electrophoretic characteristics of LPS. LPS, such as LPS from *E. coli* O55:B5 (Figure 3.7B), thereby generally displays a migration pattern where medium molecular weight ladder-like bands represent different degrees of glycosylation of the O-antigen. The lipid A attached to the core polysaccharide appears as a broad small molecular weight band (Lei *et al.*, 1991). In contrast, some LPSs have no O-antigen, and LPS from *K. pneumoniae* K52 is one example. Consequently, LPS K52 banding pattern on SDS-PAGE (Figure 3.7B) shows the migration of a broad band of small molecular weight molecules, which consist of the lipid A plus core polysaccharide moiety only.

3.3.2 Effect of different lipopolysaccharides on antigen-specific $CD8^+$ T cell responses

To test the ability of different LPSs to induce antigen-specific $CD8^+$ T cell responses, MHC I-restricted antigen-specific cytotoxicity was assessed *in vivo*. C57Bl/6 mice were injected i.m. with OVA alone or in combination with various LPSs. An *in vivo* CTL assay was performed nine days after immunisation (Figure 3.8). Results are expressed as percentage of SIINFEKL-specific lysis of target cells. No lysis was triggered in mice injected with OVA alone. With the exception of LPS from *P. gingivalis*, all LPSs were able to induce SIINFEKL-specific lysis: 31.4% ± 11.7 with LPS from *E. coli*, 51.4% ± 19.9 with LPS from *K. pneumoniae*, and 46.3% ± 20.6 with LPS from *N. meningitidis*.

In summary, LPSs from other bacteria species than *E. coli*, such as *K. pneumoniae*, and *N. meningitidis*, also have the capacity to elicit functional antigen-specific $CD8^+$ T cell responses. However, not all LPSs share that adjuvant property, since LPS from *P. gingivalis* was not able to induce antigen-specific $CD8^+$ T cell responses.

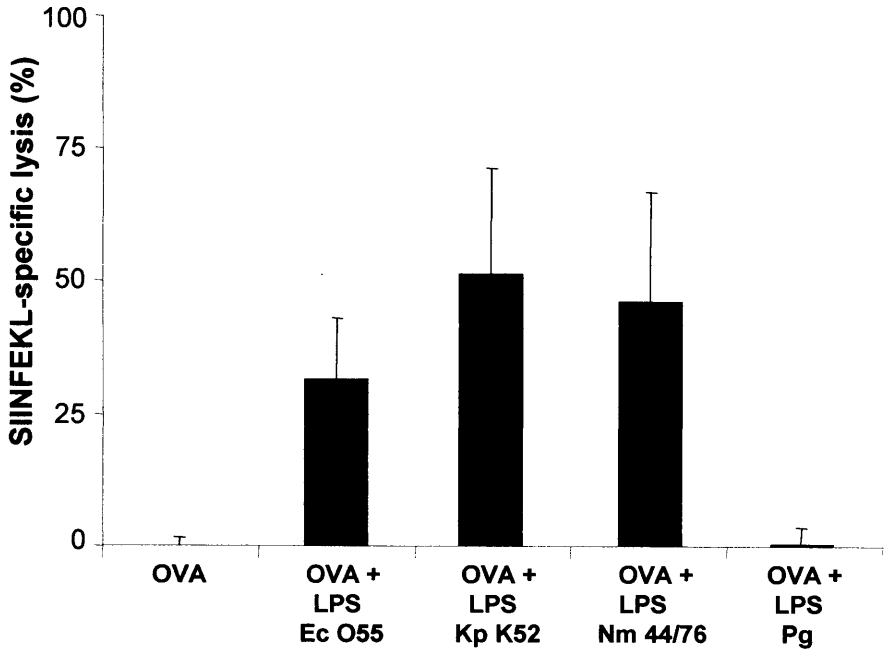

Figure 3.8. Lipopolysaccharides from different bacteria species induce functional cross-priming. C57Bl/6 mice were injected i.m. with 500 μg OVA alone or OVA in combination with LPS from *E. coli* O55:B5 (Ec 055) (10 μg), LPS from *K. pneumoniae* K52 (Kp K52) (10 μg), LPS from *N. meningitidis* 44/76 (Nm 44/76) (10 μg) or LPS from *P. gingivalis* (Pg) (10 μg). Nine days after immunisation, OVA-specific $CD8^+$ T cell cytotoxic activity was determined by *in vivo* CTL assay. Results are expressed as percentage of SIINFEKL-specific lysis. Data are represented by mean ± SD for three mice per group.

3.4 Conclusions and discussion

The aim of the studies in this chapter was to characterise further the effect of individual TLR stimulus on antigen-specific humoral and cellular responses, in a study where a range a representative TLR agonists would be assessed in parallel, using the same model.

Results demonstrated that all the TLR agonists tested were able to enhance specific IgG antibody responses to a soluble protein antigen, but that only some agonists were able to induce specific $CD8^+$ T cell responses against an exogenous soluble protein antigen.

Zymosan, poly(I:C) and CpG DNA were efficient humoral adjuvants. Firstly, they greatly enhanced the primary IgG response to a protein antigen. The response they stimulated was characterised by the production of most IgG isotypes, and in particular all three agonists increased levels of antigen-specific IgG2a antibodies. Secondly, zymosan, poly(I:C) and CpG DNA enhanced and maintained long-term IgG responses, since antigen-specific IgG antibodies of at least three different isotypes, including IgG2a and IgG2b, were still present two months after initial priming, without additional injection of antigen or adjuvant. Finally, zymosan, poly(I:C) and CpG DNA stimulated the generation of memory IgG responses. Indeed, five days after rechallenge with antigen only, antigen-specific antibody responses were enhanced, compared to responses before rechallenge, in mice that had been immunised once, two months earlier, with TLR agonist + antigen. Antibody responses were measured within five days after rechallenge, in order to exclude the contribution of antibodies produced by newly primed B cells. Enhancement of antigen-specific IgG2a responses was again a common feature.

In comparison with work related to the adjuvanticity of CpG DNA, few studies have reported on the effect of zymosan and poly(I:C) on the enhancement of antigen-specific antibody responses. Results presented here however confirm that zymosan and poly(I:C) augmented IgG2a and IgG1 responses (Wilder *et al.*, 1996; Ara *et al.*, 2001; Le Bon *et al.*, 2001).

The two CpG DNA sequences used belong to two distinct classes. CpG 2216 is a D-type sequence (A-class), which is characterised by a mixed phosphodiester-phosphorothioate oligodeoxynucleotide backbone. CpG 1668 is a K-type sequence

(B-class), which has a phophorothioate backbone. A-class CpG sequences have been reported to stimulate plasmacytoid DC and NK cells functions in particular (Ballas *et al.*, 1996; Krug *et al.*, 2001), while B-class CpG sequences have been reported to primarily stimulate B cell proliferation (Krieg *et al.*, 1995). In contrast to the statement that A-class CpG DNA (CpG 2216) stimulate weaker antigen-specific antibody responses than B-class CpG DNA (CpG 1668) (Krieg, 2002), both CpG DNA sequences were found here to have similar potent effects on antibody responses against CGG. CpG 2216 even promoted a broader range of IgG isotypes. It is possible that A-class CpG DNA poly(G) motifs, which stabilise A-class CpG DNA conformation (Kerkmann *et al.*, 2005) and may mediate CpG DNA binding to various receptors and chaperones (Verthelyi *et al.*, 2003; Kerkmann *et al.*, 2005), may be responsible for CpG DNA increased endosomal retention (Honda *et al.*, 2005a), which increases the stimulation of MyD88-IRF-7 signalling. This may represent an advantage for CpG 2216 in enhancing antigen-specific antibody responses and isotype switching.

LPS and R-848 were also able to enhance primary and memory antigen-specific IgG responses and both promoted the production of IgG2a antibodies, as opposed to peptidoglycan, which only weakly enhanced antibody responses against the protein antigen. Augmented antigen-specific IgG1, IgG2a and IgG2b titres in response to peptidoglycan have been reported, but three weekly-apart injections of peptidoglycan were required (Tomasic *et al.*, 2000). If TLR2 is involved in responses to peptidoglycan (Netea *et al.*, 2004; Watanabe *et al.*, 2004), then differences observed between the adjuvant effects of zymosan and peptidoglycan on the enhancement of antigen-specific antibody responses may show that TLR are able to differentially respond to stimuli, probably through different combinations of cooperating receptors, such as TLR2 with dectin-1 for detection of zymosan, and possibly TLR2 with Nod-2 for detection of peptidoglycan from Gram-positive bacteria (Brown *et al.*, 2003; Gantner *et al.*, 2003a; Girardin *et al.*, 2003).

The adjuvant activity of zymosan, poly(I:C), LPS, R-848 and CpG DNA on the enhancement of antigen-specific IgG responses was characterised by the promotion of various isotypes, which have different functions, although all isotypes generally contribute to preventing harmful attachment of the antigen-carrier to cells by neutralising the antigen. Every agonist promoted the production of antigen-specific

IgG2a antibodies, which are characterised by a particular capacity to induce antibody-mediated cellular cytotoxicity (ADCC) by NK cells and macrophages (Adams *et al.*, 1984; Herlyn *et al.*, 1985; Koh *et al.*, 2000). IgG2a antibodies, along with IgG1, can also induce complement-mediated cytotoxicity (CMC) (Klaus *et al.*, 1979; Herlyn *et al.*, 1985; Sensel *et al.*, 1997), and are efficient activators of the complement classical pathway, which assists antigen removal. Zymosan and soluble yeast β-glucan, for instance, were shown to induce CMC-dependent tumour rejection in a mouse model (Yan *et al.*, 1999; Hong *et al.*, 2003). Antigen elimination is also helped by IgG2a and IgG1 acting as opsonising antibodies, which can target the antigen to phagocytes. Promotion of antigen-specific antibody responses that mediate ADCC, CMC and/or pathogen neutralisation contributes to protection against pathogenesis. For example, the adjuvant effect of LPS on enhancement of antibody responses was applied to improve protection against bacterial and viral diseases in mouse models (Berinstein *et al.*, 1993; Nelson *et al.*, 2004). The adjuvant effect of CpG DNA on enhancement of specific IgG2a antibodies, against both infectious agents and tumours, has been reported in many studies (Weiner *et al.*, 1997; Kwant *et al.*, 2004; Hayashi *et al.*, 2005; Shi *et al.*, 2005b). Interestingly, although CpG 2216 and CpG 1668 were found to enhance antigen-specific IgG responses similarly, differences in which effector cell type are activated for ADCC have been reported: in the presence of A-class CpG, ADCC was mediated by NK cells, while both NK cells and granulocytes were effectors in the presence B-class CpG (van Ojik *et al.*, 2003).

As mentioned above, zymosan, poly(I:C), LPS, R-848 and CpG DNA were able to enhance antigen-specific primary and memory IgG responses. Such stimulation of specific B cell responses suggests that the agonists provided signals during initial priming that were integrated by immune cells and translated into B cell activation and antibody class switching, but also stimulated B cell differentiation into memory B cells and in some cases long-lived antibody secreting plasma cells (Manz *et al.*, 2005; Shapiro-Shelef *et al.*, 2005). Cognate $CD4^+$ T cell help is known to support B cell responses through CD40L/CD40 interactions (Castigli *et al.*, 1994; Ahonen *et al.*, 2002; Eaton *et al.*, 2004). This suggests that agonists may have also enhanced the generation of antigen-specific $CD4^+$ T cells, and promoted Th1 responses that stimulated the production of antigen-specific antibodies of the IgG2a isotype. Zymosan, LPS from *E. coli* and R-848 for example were found to increase antigen-

specific $CD4^+$ T cell responses, which in the case of LPS were directly proven to provide help for antigen-specific IgG2a antibody production (Pape *et al.*, 1997; Vasilakos *et al.*, 2000; Ara *et al.*, 2001; Wille-Reece *et al.*, 2005).

Other cell types may contribute to promoting isotype-switching. Activation of NK cells by poly(I:C) can increase antigen-specific IgG1 and IgG2a responses (Wilder *et al.*, 1996), possibly through IFN-γ secretion (Finkelman *et al.*, 1988; Amigorena *et al.*, 1990). Indeed, antigen-specific IgG2a antibody production in response to R-848 and CpG DNA for example has been shown to be dependent on IFN-γ (Vasilakos *et al.*, 2000; Van Uden *et al.*, 2001), and DC-NK cells cross-talk may stimulate IFN-γ production, through cytokines such as IFN-α/β, IL-18 and IL-12 (Van Uden *et al.*, 2001; Kamath *et al.*, 2005). Induction of antigen-specific IgG2a production by microbial products such as CFA, poly(I:C), R-848 and CpG DNA was found to be IFN-α/β-dependent, and IFN-α/β were shown to promote antigen-specific isotype switching and long-term and memory antibody responses (Finkelman *et al.*, 1991; Vasilakos *et al.*, 2000; Le Bon *et al.*, 2001; Van Uden *et al.*, 2001). Direct DC-B cell interactions may also promote antigen-specific IgG2a responses (Wykes *et al.*, 1998). There is also evidence that direct B cell activation by PAMPs, more specifically CpG DNA, could promote IgG2a isotype switching, through TLR activation, TLR9 in the case of CpG DNA (Peng *et al.*, 2003; Lin *et al.*, 2004).

Adjuvant activities of LPS from *E. coli* on enhancement of antigen-specific antibody responses were found to be dependent on TLR4 (Skidmore *et al.*, 1976), but the contribution of other TLR receptors, such as TLR2, TLR3 and TLR7 in the adjuvant effect of zymosan, poly(I:C) and R-848, respectively, remains to be assessed.

Zymosan, poly(I:C), LPS from *E. coli*, R-848 and CpG DNA are PAMPs from various microbial origin. The present study demonstrated that these TLR agonists, even though known to stimulate innate responses through activation of different TLRs, were all able to enhance antigen-specific humoral responses. Mechanisms controlling the adjuvant activities of the different TLR agonists on antigen-specific antibody responses would need to be investigated, in order to understand how the immune system translates the detection of various microbial stimuli into a particular adaptive immune response.

Immune responses against pathogens are mediated by both humoral and cellular adaptive responses. Having determined that agonists of a range of TLRs were able to enhance antigen-specific antibody responses, the effect of various TLR agonists on the *in vivo* generation of antigen-specific $CD8^+$ T cell responses, focussing on induction of cross-priming against a soluble protein antigen, was also assessed.

Results showed that functional $CD8^+$ T cell responses were generated against OVA when OVA was administered in the presence of poly(I:C), CpG DNA and to a lesser extent LPS from *E. coli*. Since cross-tolerance normally develops against cross-presented exogenous antigens (Kurts *et al.*, 1997; Bonifaz *et al.*, 2002), induction of cross-priming in the presence of some TLR stimuli demonstrated that these agonists were able to stimulate immune responses that licensed the generation of functional antigen-specific CTL responses. Indeed, some microbial products and bacteria were found to overcome the induction of cross-tolerance (Mazzaccaro *et al.*, 1996; Bennett *et al.*, 1997; Simmons *et al.*, 1999).

Results confirmed the adjuvant activity of CpG DNA on the induction of cross-priming (Cho *et al.*, 2000). Both A- and B-class CpG DNA sequences induced functional antigen-specific $CD8^+$ T cells, and contrary to what was reported (Krieg, 2002), CpG 2216 (A-class) was not found to promote stronger CTL activity than CpG 1668 (B-class).

LPS from *E. coli*, but also LPS from other enterobacteria, such as *K. pneumoniae* and *N. meningitidis*, were found to induce cross-priming. In contrast, LPS from *P. gingivalis*, was unable to stimulate the generation of antigen-specific $CD8^+$ T cell responses. Differences in the effect of LPSs on the immune system had been observed, where LPS from *P. gingivalis* stimulated responses in C3H/HeJ mice (Joiner *et al.*, 1982), which are hyporesponsive to LPS from many bacteria, including *E. coli* and *Salmonella* spp. (Bradford Hill *et al.*, 1940; Sultzer, 1968). LPS from *P. gingivalis* and other bacteria, such as *L. interrogans*, activate intracellular immune pathways and cytokine expression patterns different to those triggered by LPS from *E. coli* (Hirschfeld *et al.*, 2001; Werts *et al.*, 2001). Interestingly, in a model of adoptive transfer of $CD8^+$ TCR transgenic T cells, LPS from *E. coli* was also shown to promote secretion of IFN-γ, from antigen-specific $CD8^+$ T cells, after antigen-restimulation *in vitro*, while LPS from *P. gingivalis* was unable to do so (Pulendran *et al.*, 2001). Those data reflect the fact that LPS from *P. gingivalis* stimulates the

activation of immune programs that may not support the development of adaptive responses, which may explain its inability to induce cross-priming.

In fact, it was demonstrated that LPS from bacteria such as *E. coli* and *N. meningitidis* stimulated cytokine responses through activation of TLR4, while LPS from *P. gingivalis* activated TLR2 (Pulendran *et al.*, 2001; Zughaier *et al.*, 2004). TLR2 agonists for instance fail to induce IFN-α/β (Toshchakov *et al.*, 2002; Siren *et al.*, 2005), which is one of the stimuli that licenses the induction of cross-priming (Le Bon *et al.*, 2003). Therefore it is possible that, in a similar way to LPS from *P. gingivalis*, zymosan and peptidoglycan activate intracellular immune pathways that cannot translate into the generation of $CD8^+$ T cell responses against an exogenous antigen. R-848 is a strong immunomodulator that can stimulate innate and Th1 cytokine production, and lymphocyte activation (Ahonen *et al.*, 1999; Wagner *et al.*, 1999; Bishop *et al.*, 2000; Caron *et al.*, 2005; Gautier *et al.*, 2005). R-848 was however unable to induce cross-priming against a soluble protein, when co-injected once with the antigen. The effect of R-848 and other imidazoquinolines on induction of cross-priming have been tested since and R-848, even when co-administered with the antigen three times, was found to be a poor inducer of antigen-specific $CD8^+$ T cell responses (Wille-Reece *et al.*, 2005), while imidazoquinoline S-27609 could only enhance the adjuvant effect of anti-CD40 stimuli (Ahonen *et al.*, 2004). R-848 lack of efficiency in inducing cross-priming may be due to the lack of expression of TLR7 on murine $CD8^+$ DC (Edwards *et al.*, 2003), which are thought to play an important role in cross-priming (den Haan *et al.*, 2000). R-848 and other TLR7 stimuli may in fact promote innate immune programs that favour IL-12-mediated $CD4^+$ T cell activation and Th1 responses that stimulate antibody- and non-$CD8^+$ T cell-mediated responses. A new compound was reported to be able to induce cross-priming, however it was conjugated to the antigen and injected three times. It was proposed that conjugates may have mediated cross-priming by activating other DC types than $CD8^+$ DC, such as myeloid and/or plasmacytoid DC (Wille-Reece *et al.*, 2005).

Zymosan, poly(I:C), LPSs from the three enterobacteria species tested, R-848 and CpG DNA were thus shown to enhance adaptive immune responses. The effect of some agonists, such as zymosan and R-848 were restricted to the enhancement of

antigen-specific humoral responses, while LPS, poly(I:C) and CpG DNA were also able to induce $CD8^+$ T cell responses against an exogenous soluble protein antigen. Overall, these agonists augmented particular responses that would promote cytotoxic mechanisms, both $CD8^+$ T cell- and non-$CD8^+$ T cell mediated. These TLR stimuli thereby displayed properties that make them potential adjuvants against intracellular pathogens in particular, against which cytotoxic mechanisms are essential.

Immune pathways that trigger the licensing of cross-priming are still being investigated. IFN-α/β, which play an important role in activation of immune pathways by poly(I:C), LPS and CpG DNA, have been shown to be a major cross-priming licensing stimulus. In order to characterise how the immune system translates TLR agonist stimulation into cross-priming licensing, the contribution of the IFN-α/β signalling pathway in induction of cross-priming by poly(I:C), LPS from enterobacteria, and CpG DNA was assessed, and results are presented in Chapter 5.

Chapter 4

Effects of high-mannose carbohydrate structures on adaptive immune responses

4.1 Introduction

Carbohydrates make up most of organic matter and form major structural components of microorganisms such as bacteria, yeast and viruses. Polysaccharides were long thought rather innocuous, yet interest in studying the immunological contribution of carbohydrate fractions from pathogens developed; and the antigenicity of many polysaccharides was established. For example, capsular polysaccharides of bacteria such as pneumococci and meningococci (Bruyn *et al.*, 1992; Mandrell *et al.*, 1995), and *Candida albicans* cell wall polysaccharides (Cutler, 2005) induce opsonising and neutralising antibodies, which are critical in controlling dissemination during infection. High-density oligomannose clusters on HIV-1 gp120 are also target epitopes (Sanders *et al.*, 2002; Scanlan *et al.*, 2002).

Interestingly, certain polysaccharides have been found to display non-specific immunomodulatory activities. Yeast cell wall β-glucans for example were typically exploited, and are readily sold as dietary supplements to potentiate the immune system. Experimental studies have demonstrated that β-glucans, isolated from yeasts, bacteria and plants, non-specifically enhance humoral and cellular responses. Indeed it has been reported that β-glucans stimulate cytokine production (Sherwood *et al.*, 1987; Abel *et al.*, 1992; Lowe *et al.*, 2002), complement activation (Schenkein *et al.*, 1981; Glovsky *et al.*, 1983), phagocytosis (Yun *et al.*, 2003), lysosomal enzyme activity (Sakurai *et al.*, 1997) and increase resistance to some bacterial infections (Williams *et al.*, 1983; Estrada *et al.*, 1997; Kournikakis *et al.*, 2003; Li *et al.*, 2004; Rice *et al.*, 2005). Immunostimulatory properties of other glucans, such as chitosan, are also being investigated (Shibata *et al.*, 1997; Choi *et al.*, 2001; Feng *et al.*, 2004). Bacterial capsular polysaccharides, isolated from *Bacteroides fragilis* or *Klebsiella pneumoniae* for example, also exhibit potent immunostimulatory properties (Ho *et al.*, 2000; Tzianabos *et al.*, 2000).

Many innate receptors that are crucial for controlling adaptive immune responses have glycoside specificities. Carbohydrates thereby benefit from an exceptional relationship with the immune system, and some are being considered as potential adjuvants to improve the immunogenicity of vaccines (Maeda *et al.*, 2004; Silva *et al.*, 2004; Hasegawa *et al.*, 2005). Mannose oligosaccharides, for example, are common ligands for a number of lectins that are involved in the innate immune response, including mannose-binding lectin MBL and the mannose receptor, and high-mannose molecules such as yeast mannan have been shown to modulate immune responses. *In vitro* activation of human B cells by mannan has been reported (Mangeney *et al.*, 1989). Mannan added to human peripheral blood mononuclear cells was also shown to stimulate proliferation, as measured by ^{3}H-thymidine incorporation (Ausiello *et al.*, 1986; Durandy *et al.*, 1986; Podzorski *et al.*, 1990; Savolainen *et al.*, 2003). Mice injected intraperitoneally with mannan showed an increase in number of peritoneal macrophages and polymorphonuclear cells, and proliferation of Kupffer cells, which are resident macrophages of the liver that participate in the clearance of organisms from the bloodstream by phagocytosis and through the initiation of inflammatory processes (Okawa *et al.*, 1986; Gregory *et al.*, 2002; Okawa *et al.*, 2002). Expression of various cytokines in response to mannan has been detected *in vivo* (Wang *et al.*, 1998). Mannan also induces the production of pro-inflammatory cytokine TNF-α (Garner *et al.*, 1996), of IL-2, as well as both IFN-γ and IL-4 (Li *et al.*, 1998). These observations have prompted investigations into the use of mannan and mannosides as glycoconjugate adjuvants, to enhance adaptive immune responses against microbial and tumour antigens by specifically targeting lectin receptors (Okawa *et al.*, 1992; Toda *et al.*, 1997; Shiku *et al.*, 2000; Berlyn *et al.*, 2001; Stambas *et al.*, 2005).

A series of studies in particular demonstrated that mannan conjugated to mucin tumour antigen MUC1 could induce strong Th1 or Th2 type immune responses, depending on the conjugation protocol. Mannan coupled to MUC1 under oxidising conditions induced complete tumour protection in mice, with antigen-specific responses that were characterised by high CTL precursor frequency, IL-12 and high IFN-γ secretion and a predominant IgG2a antibody response (Apostolopoulos *et al.*, 1995; Apostolopoulos *et al.*, 1996; Lofthouse *et al.*, 1997; McKenzie *et al.*, 1998; Lees *et al.*, 1999; Pietersz *et al.*, 2000). In contrast, MUC1 conjugated to mannan

under reducing conditions induced Th2 responses, characterised by secretion of IL-4 and IL-10 but no IFN-γ, and a predominant IgG1 antibody response. Prophylaxis studies were extended into primate models (Vaughan *et al.*, 1999; Vaughan *et al.*, 2000), while results from clinical trials with cancer patients showed some increased anti-MUC1 antibody titres, T-cell proliferation and CTL responses (Apostolopoulos *et al.*, 1997; Apostolopoulos *et al.*, 2000).

The literature suggests that mannan-conjugates have adjuvant potential for the generation of antigen-specific humoral and cellular responses. Studies investigating the effectiveness of modified-mannan conjugates, in enhancing cytotoxic T-cell activity especially, have focussed on inducing responses against one particular antigen, mucin MUC1. Conjugation of oxidised mannan to other peptide antigens has only been mentioned briefly (McKenzie *et al.*, 1998). It was therefore of interest to explore further the ability of mannan, conjugated to or co-administered with other antigens, to promote functional antigen-specific cellular and humoral responses.

The work presented in this chapter addresses the following question: Do mannan, modified-mannan conjugates and more generally high-mannose structures exhibit a consistent ability to generate adaptive immune responses against an exogenous soluble antigen?

4.2 Effect of modified-mannan protein conjugates on induction of antigen-specific $CD8^+$ T cell responses

Studies published by Apostolopoulo and McKenzie demonstrated that oxidised-mannan conjugated to mucin MUC1 produced strong CTL responses *in vitro* and led to tumour rejection, in mice immunised with three intraperitoneal (i.p) doses of conjugates. Firstly, in order to verify whether the adjuvant effect of modified-mannan was applicable to any other protein antigen, OVA, a well characterised antigen, was used. Secondly, in order to analyse the elementary adjuvant influence of mannan on promoting primary responses, single injection protocols were carried out. Finally, intramuscular injection was chosen, as a route relevant for vaccination.

The aim of the work presented below was to examine whether oxidised-mannan and reduced-mannan, when conjugated to OVA, could induce functional primary $CD8^+$ T cell responses against OVA, after a single intramuscular injection. C57Bl/6 mice

were injected i.m. with OVA alone, or OVA in combination with mannan from *Saccharomyces cerevisiae*, or OVA conjugated to oxidised-mannan (OVA-oxMan) or reduced-mannan (OVA-redMan) (see 2.3.4.1).

To first assess whether modified-mannan-OVA conjugates were able to induce proliferation of OVA-specific $CD8^+$ T cells, the frequency of $CD8^+$ T cell bearing SIINFEKL-specific TCR was measured in spleen eight days after immunisation, using H-$2K^b$-SIINFEKL tetramer staining followed by FACS analysis (see 2.6.1). Representative FACS analysis dot plots are shown in Figure 4.1A. No tetramer-positive (Tet^+) cells could be detected in naïve mice or mice injected with OVA (Figure 4.1B). Surprisingly, neither OVA-oxMan nor OVA-redMan could induce any detectable OVA-specific $CD8^+$ T cell population, while co-administration of intact mannan with OVA induced expansion of SIINFEKL-specific $CD8^+$ T cells (0.7 % Tet^+ ± 0.5).

In order to determine whether SIINFEKL-specific $CD8^+$ T cells, generated in the presence of mannan-OVA conjugates, had the characteristics of effector cells, their ability to produce IFN-γ was assessed, using IFN-γ ELISPOT assay, as described previously. Eight days after immunisation, the ability of SIINFEKL-specific splenic $CD8^+$ T cells to produce IFN-γ upon restimulation *in vitro* was determined and the frequency (*f*) of OVA-specific $CD8^+$ T cells was expressed as a number of IFN-γ spots per 10^6 $CD8^+$ T cells (Figure 4.1C). No spots could be detected after restimulation in the absence of SIINFEKL peptide. OVA-oxMan (11 spots ± 4, f=141 ± 49) and OVA-redMan (16 spots ± 6, f=208 ± 80) were not able to induce an increase in number of spots, in comparison to naïve mice (8 spots ± 4, f=96 ± 41) or mice injected with OVA alone (18 spots ± 3, f=214 ± 38). In contrast, free native mannan stimulated the generation of IFN-γ-producing OVA-specific $CD8^+$ T cells (110 spots ± 75, f=1338 ± 905).

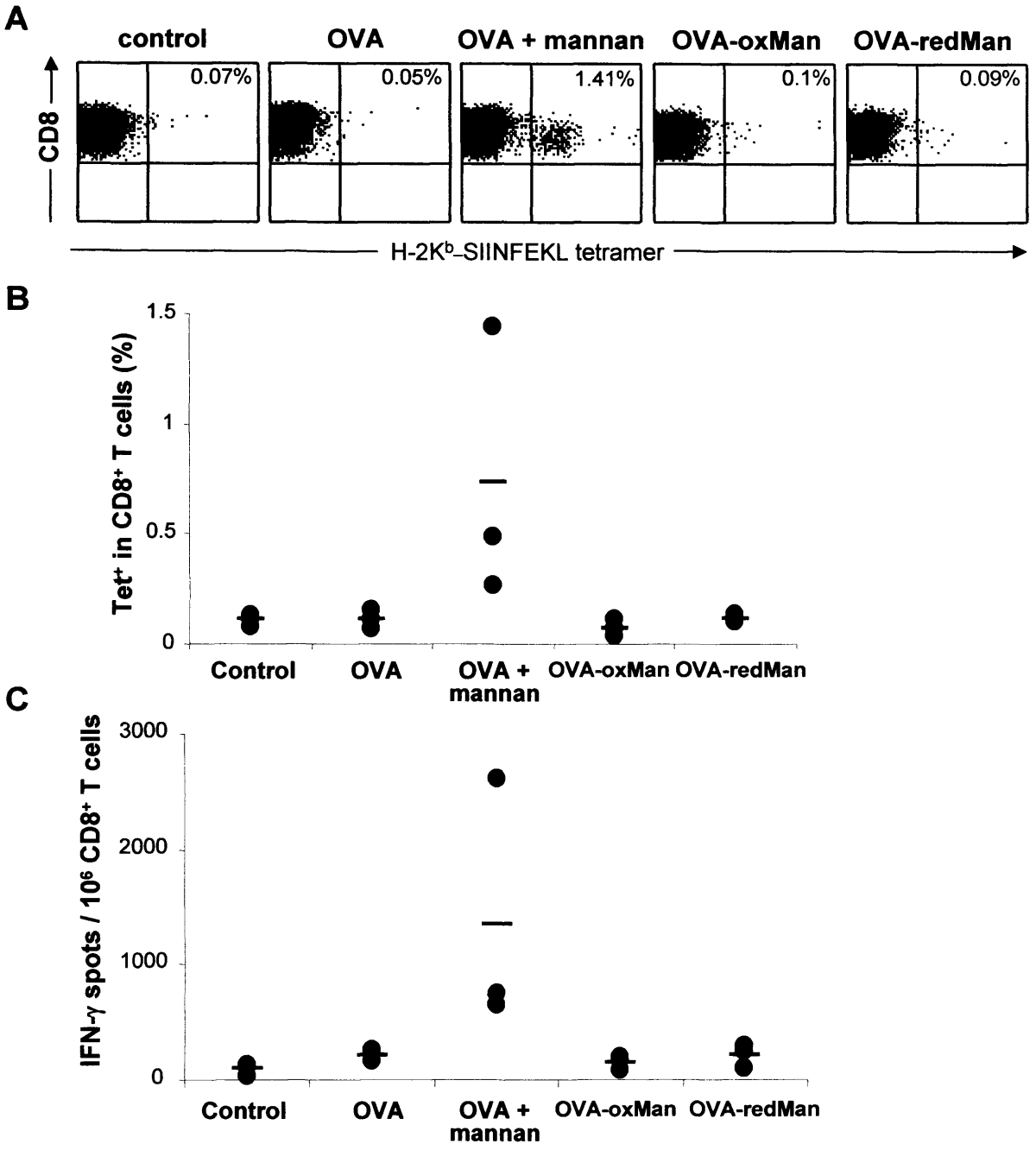

Figure 4.1 Modified-mannan OVA conjugates are not able to generate OVA-specific CD8$^+$ T cells. C57Bl/6 mice were injected i.m. with 500 μg ovalbumin (OVA) alone, OVA in combination with mannan from *S. cerevisiae* (2.8 mg), periodate-oxidised mannan conjugated to OVA (OVA-oxMan) or sodium borohydride-reduced mannan-Ova conjugates (OVA-redMan); OVA-mannan conjugates contained 500 μg OVA and 2.8 mg mannan. (A, B) Eight days after immunisation, SIINFEKL-specific CD8$^+$ T cells in spleens were quantified using K^b-SIINFEKL tetramer staining. (A) Representative FACS dot plots of tetramer staining. The value in the right corner of each panel represents the percentage of tetramer-positive (Tet$^+$) cells among CD8$^+$ T cells. (B) Percentage of Tet$^+$ cells, gated on CD8$^+$ T cells, for three individual mice per group; horizontal lines represent the mean percentage of Tet$^+$ cells for each group. (C) Eight days after immunisation, splenic CD8$^+$ T cells were assessed for SIINFEKL-specific IFN-γ secretion by ELISPOT assay. Results are expressed as the number of spots per 10^6 CD8$^+$ T cells for three individual mice per group; horizontal lines represent the mean number of spots for each group.

Results from tetramer staining and ELISPOT assays correlated and showed that modified-mannan conjugated to OVA, delivered once intramuscularly, failed to increase the frequency of OVA-specific $CD8^+$ T cells. Co-administration of OVA and native mannan, however, induced expansion and differentiation of OVA-specific $CD8^+$ T cells.

Oxidised-mannan MUC1 conjugates were shown to target the MHC I presentation pathway, leading to cross-priming against MUC1, due to the presence of aldehydes from oxidised-mannan (Apostolopoulos *et al.*, 2000). Other aldehyde-containing adjuvants have sparked interest, and saponin QS-21, for example, co-administered with OVA was found to be as effective as OVA-QS-21 conjugates in inducing OVA–specific cytotoxic T-lymphocytes (Soltysik *et al.*, 1995).

Therefore, the possibility that, in the present model, modified-mannan molecules required to be unconjugated to OVA to be able to induce $CD8^+$ T cell responses was examined. To that effect, C57Bl/6 mice were injected i.m. with OVA alone, or OVA in combination with mannan, oxidised-mannan, or reduced-mannan. The adjuvant capacity of intact and modified-mannan to generate OVA-specific $CD8^+$ T cell responses was assessed in spleen using tetramer staining and IFN-γ ELISPOT assay eight days after immunisation. Tetramer staining (Figure 4.2A) was performed on splenic $CD8^+$ T cells. Immunisation with OVA + oxMan or OVA + redMan did not result in the generation of an OVA-specific $CD8^+$ T cell population. Consistent with results shown in Figure 4.1, an average of 0.8% of $CD8^+$ T cells were Tet^+ in mice immunised with OVA + mannan. IFN-γ ELISPOT assay (Figure 4.2B) was performed with splenic $CD8^+$ T cells. Ox-Man and red-Man were not able to increase the frequency of IFN-γ-producing SIINFEKL-specific $CD8^+$ T cells. Results confirmed firstly that mannan co-administered with OVA induced an increase in the frequency of OVA-specific-$CD8^+$ T cells able to produce IFN-γ (f=1410 ± 445, compared to f=191 ± 26 with OVA alone), and secondly that OVA-specific $CD8^+$ T cells generated in the presence of native mannan had the capacity to become effector cells.

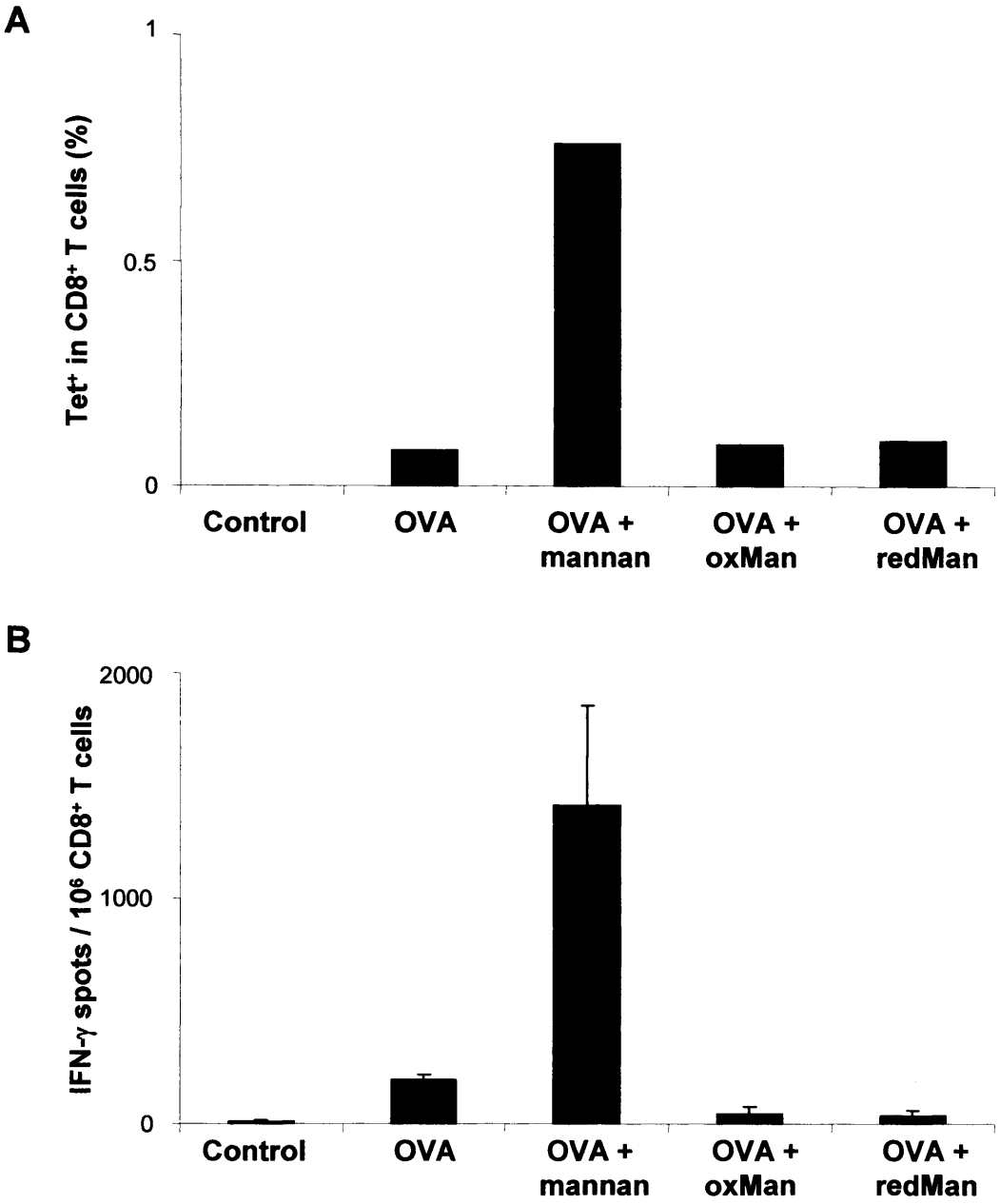

Figure 4.2. Mannan, but not modified-mannan, induces functional cross-priming against OVA. C57Bl/6 mice were injected i.m. with 500 μg OVA alone or OVA in combination with mannan from *S. cerevisiae* (2.8 mg), periodate-oxidised mannan (oxMan) (2.8 mg) or sodium borohydride-reduced mannan (redMan) (2.8 mg). (A) Eight days after immunisation, SIINFEKL-specific $CD8^+$ T cells in spleens were quantified using K^b-SIINFEKL tetramer staining Results are expressed as percentage of tetramer-positive (Tet^+) cells, gated on $CD8^+$ T cells, in pooled splenocytes from three mice per group. (B) Eight days after immunisation, splenic $CD8^+$ T cells were assessed for SIINFEKL-specific IFN-γ secretion by ELISPOT assay. Results are expressed as the number of spots per 10^6 $CD8^+$ T cells, which were purified from pooled splenocytes from three mice per group. Data are represented by mean ± SD for three ELISPOT wells per group.

In summary, oxidised- or reduced-mannan, either conjugated to OVA or co-administered with OVA, were not able to elicit antigen-specific $CD8^+$ T cell responses. Surprisingly, free mannan exhibited adjuvant properties, in inducing expansion and differentiation of OVA-specific $CD8^+$ T cells.

A commercial preparation of mannan was used in this study. Specificity was confirmed by the manufacturer, according to optical rotation (over +73°, comparable to rotation values described by (Masuoka, 2004)) and thin-layer chromatography. Nonetheless, the effect of potential β-glucan contaminants was verified, and highly purified β-glucans, co-administered with OVA, was not able to induce Kb-SIINFEKL-positive $CD8^+$ T cells.

The finding of this intriguing property of mannan prompted further studies, which focussed on native mannan, in an attempt to investigate in more detail a feature of free mannan that had not been reported before. Therefore, the ability of free mannan to induce antigen-specific adaptive immune responses was characterised further. Mannan is a polymannose component of yeast cell wall; in order to assess the adjuvant capabilities of high-mannose molecules of organisms that belong to other kingdoms, structures originating from bacteria and viruses were included in the study.

4.3 Induction of antigen-specific cellular responses by mannan and polymannose structures

4.3.1 Antigen-specific $CD8^+$ T cell responses

4.3.1.1 Effect of mannan on induction of antigen-specific $CD8^+$ T cell responses

Mannan, co-administered with OVA, consistently indicated an ability to induce OVA-specific $CD8^+$ T cell responses. Indeed, in the presence of mannan *in vivo*, OVA-specific $CD8^+$ T cell were primed to proliferate, as measured by H-$2K^b$-SIINFEKL tetramer staining, and were shown to have differentiated into effector cells, as assessed by IFN-γ ELISPOT assays.

To characterise further the functional properties of OVA-specific $CD8^+$ T cells generated in the presence of mannan, the cytolytic potential of these cells was assessed. First, expression of granzyme B by OVA-specific $CD8^+$ T cells in the spleen was examined by intracellular staining (see 2.6.1) and FACS analysis. A

representative FACS analysis histogram is shown in Figure 4.3A. Cells stained with anti-granzyme B antibody presented a shift in fluorescence intensity (MFI), as cells displayed a MFI 3.4 ± 1.4 times greater than MFI of cells stained with isotype control (Figure 4.3B). This illustrated expression of granzyme B by OVA-specific $CD8^+$ T cells. Distribution of granzyme B expression, in the $CD8^+/Tet^+$ cell population, was also examined (Figure 4.3C). Results showed that 19.3% ± 10.3 of OVA-specific $CD8^+$ T cells expressed granzyme B after immunisation with OVA + mannan.

Expression of granzyme B is another indication that antigen-specific $CD8^+$ T cells, primed in the presence of mannan, are armed for effective cell killing.

To test specifically the cytotoxic function of OVA-specific $CD8^+$ T cells, *in vivo*, CTL assays were performed (see 2.6.3). Data from the *in vivo* CTL assay are presented as percentage of SIINFEKL-specific lysis (see 2.6.3.2) (Figure 4.3D). OVA injected on its own did not generate OVA–specific CTL. In mice immunised with OVA + mannan, 31.9% ± 18.6 of target cells were killed.

In summary, mannan was able to induce fully functional antigen-specific $CD8^+$ T cell responses.

4.3.1.2 Induction of antigen-specific $CD8^+$ T cell responses by high-mannose molecules

High-mannose PAMPS are ubiquitous (Prehm *et al.*, 1976; Jansson *et al.*, 1985; Geyer *et al.*, 1988; Domer *et al.*, 1989; Kohl *et al.*, 2004). Given that the adjuvant capacity of mannan was established, it was of interest to investigate whether other high-mannose microbial structures shared this property of inducing antigen-specific $CD8^+$ T cell responses.

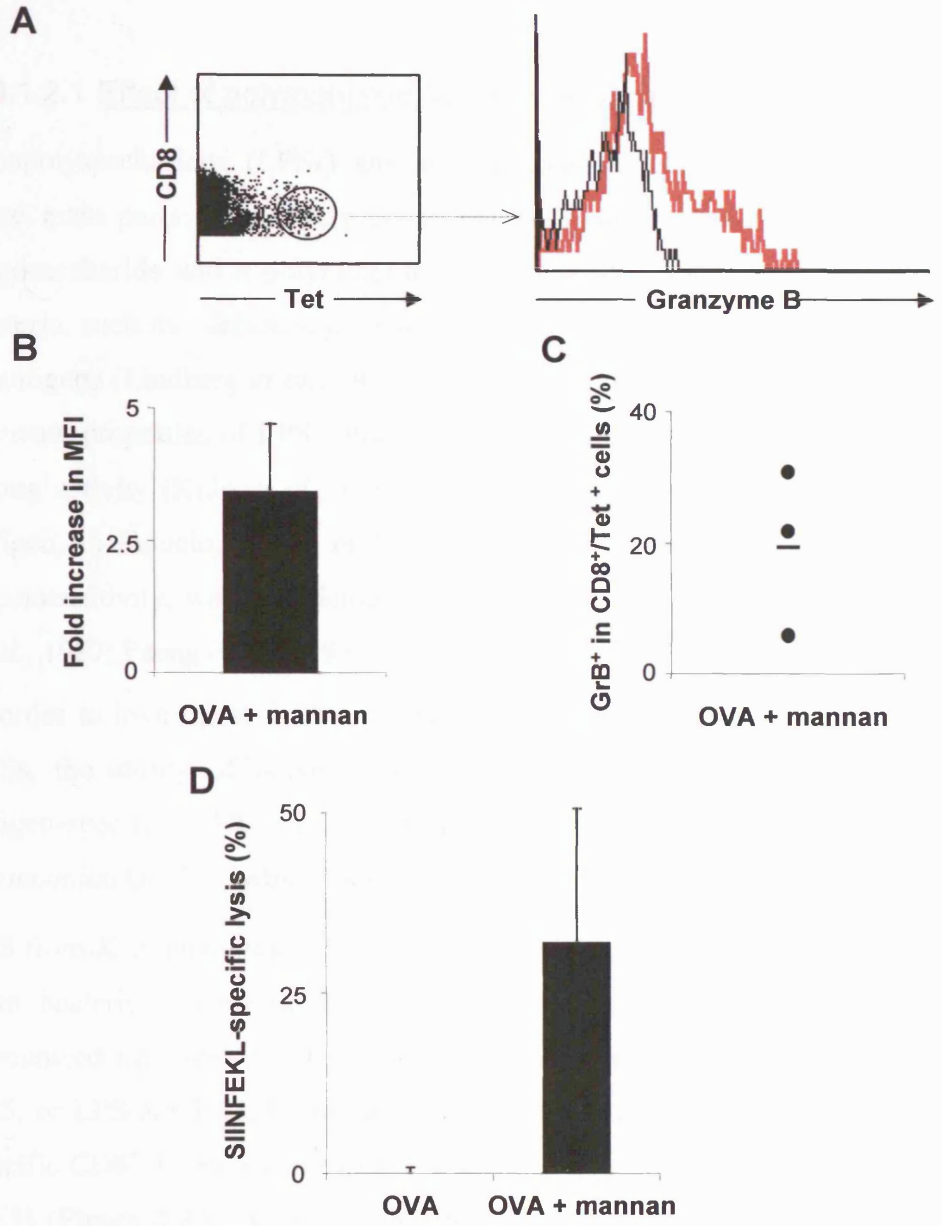

Figure 4.3. Mannan induces antigen-specific cytotoxicity *in vivo*.
C57Bl/6 (B6) mice were injected i.m. with OVA (500 µg) in combination with mannan from *S. cerevisiae* (2.8 mg). Eight days after immunisation, splenic $CD8^+$ T cells were stained with K^b-SIINFEKL tetramer and for intracellular granzyme B. (A) Representative FACS histogram of splenocytes from a mouse immunised with OVA + mannan. Splenocytes, enriched in $CD8^+$ T cells, were stained with anti-granzyme B antibody (—) or isotype control (—). Events were gated on $CD8^+$ tetramer-positive (Tet^+) T cells. (B) Results are expressed as percentage of increase in the mean fluorescence intensity (MFI) of cells stained with anti-granzyme B antibody compared to cells stained with isotype control. Data are represented by mean ± SD for three mice per group. (C) Results are expressed as percentage of granzyme B-positive cells (GrB^+), with events gated on $CD8^+$ Tet^+ cells, for three individual mice; horizontal lines represent the mean percentage of GrB^+ cells for each group. (D) B6 mice were injected i.m. with 500 µg OVA alone or OVA in combination with mannan from *S. cerevisiae* (2.8 mg). Nine days after immunisation, OVA-specific $CD8^+$ T cell cytotoxic activity was determined by *in vivo* CTL assay. Results are expressed as percentage of SIINFEKL-specific lysis. Data are represented by mean ± SD for three mice per group.

4.3.1.2.1 Effect of polymannose lipopolysaccharides

Lipopolysaccharides (LPSs) are heterogenous molecules generally consisting of three main parts: a relatively conserved phosphoglycolipid called lipid A, a core oligosaccharide and a polymorphic polysaccharide O-antigen (Raetz, 1990). Some bacteria, such as *Klebsiella pneumoniae* serotypes O3 and O5, produce polymannan O-antigens (Lindberg *et al.*, 1972; Curvall *et al.*, 1973). Early studies, analysing the adjuvant properties of LPSs, illustrated that polymannan LPSs exhibited a particular strong activity (Kido *et al.*, 1985a). Specific adjuvanticity of the high-mannose O-antigen, in inducing BSA- or OVA-specific antibody responses and delayed-type hypersensitivity, was later demonstrated (Kido *et al.*, 1985b; Ohta *et al.*, 1985; Ohta *et al.*, 1987; Paeng *et al.*, 1996).

In order to investigate further the particular adjuvant characteristics of polymannan LPSs, the ability of polymannose LPS from *K. pneumoniae* O3:K55 to induce antigen-specific $CD8^+$ T cell responses, in comparison with LPS from *K. pneumoniae* O-:K52, which lacks the O-antigen, was examined.

LPS from *K. pneumoniae* K55 (LPS Kp K55) and K52 (LPS Kp K52) were extracted from bacteria and prepared as described in paragraph 2.3.3. C57Bl/6 mice were immunised i.m. with OVA alone, or OVA in combination with mannan, LPS Kp K55, or LPS Kp K52. Eight days after immunisation the frequency of SIINFEKL-specific $CD8^+$ T cell was measured in spleen, using H-$2K^b$-SIINFEKL tetramers (see 2.6.1) (Figure 4.4A). LPS Kp K55 induced the expansion of a SIINFEKL-specific $CD8^+$ T cell population (0.5% ± 0.2), and so did LPS Kp K52 (0.14% ± 0.0, p=0.03). Mannan also stimulated expansion of OVA-specific $CD8^+$ T cells significantly (p=0.024). The cytolytic potential of these cells was assessed by conducting CTL assays *in vivo* (see 2.6.3) (Figure 4.4B). Results show that OVA + LPS Kp K55 induced 78.6% ± 1.1 SIINFEKL-specific lysis (p=0.000), and OVA + LPS Kp K52 induced 63.0% ±13.8 lysis (p=0.003). In mice immunised with OVA + mannan, 18.9% ± 7.6 targets were killed (p=0.032).

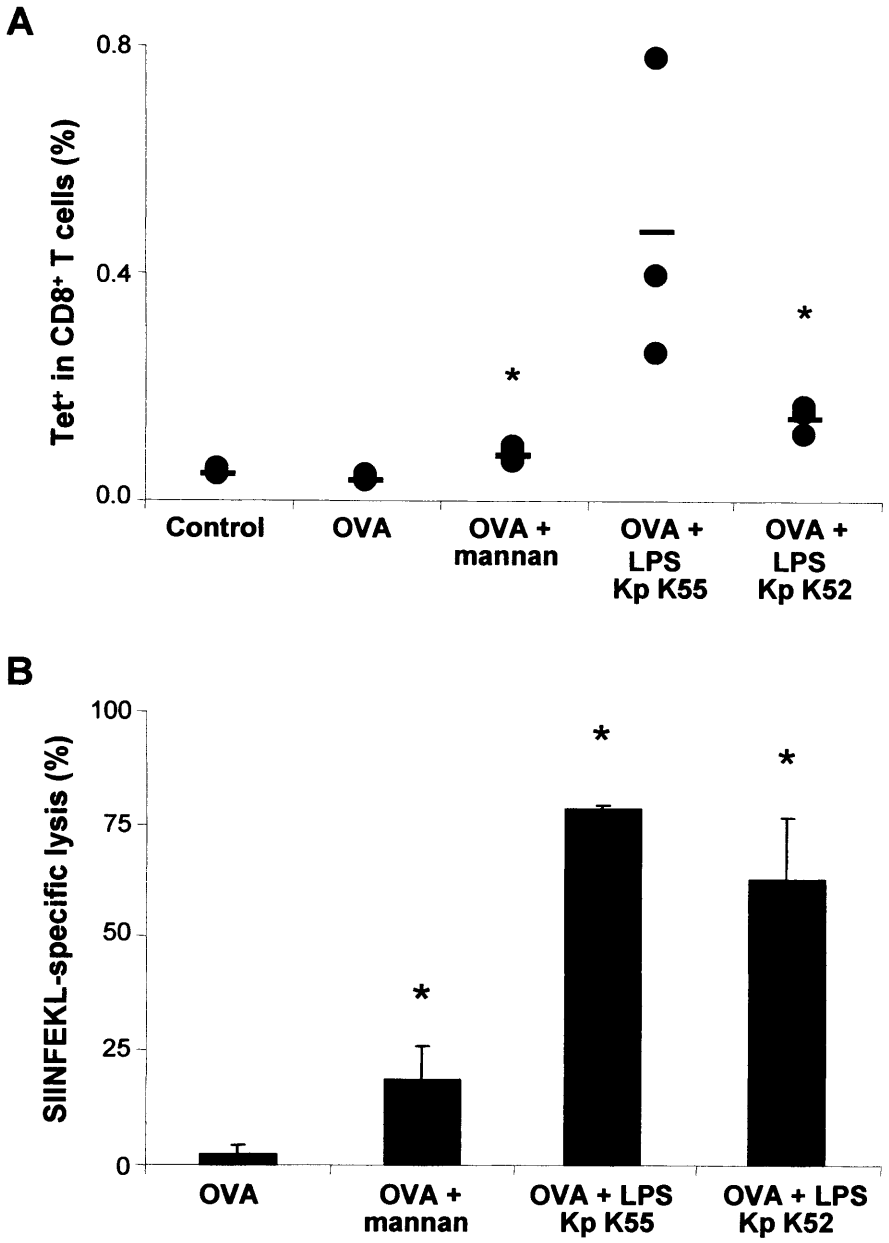

Figure 4.4. The effect of polymannose lipopolysaccharide on induction of cross-priming against OVA. C57Bl/6 mice were injected i.m. with 500 μg OVA alone or OVA in combination with mannan from *S. cerevisiae* (2.8 mg), LPS from *K. pneumoniae* O3:K55 (Kp K55) (10 μg) or LPS from *K. pneumoniae* K52 (Kp K52) (10 μg). (A) Eight days after immunisation, SIINFEKL-specific $CD8^+$ T cells in spleens were quantified using K^b-SIINFEKL tetramer staining. Results are expressed as percentage of tetramer-positive (Tet^+) cells, gated on $CD8^+$ T cells, for three individual mice per group; horizontal lines represent the mean percentage of Tet^+ $CD8^+$ cells for each group. (B) Nine days after immunisation, SIINFEKL-specific $CD8^+$ T cell cytotoxic activity was determined by *in vivo* CTL assay. Results are expressed as percentage of SIINFEKL-specific lysis. Data are represented by mean ± SD for three mice per group.
* $p<0.05$ versus control by one-way ANOVA test.

In summary, both polymannose-LPS Kp K55 and rough LPS Kp K52 were able to elicit the generation of OVA-specific $CD8^+$ T cells that were cytotoxic.

The ability of a viral high-mannose protein to induce antigen specific $CD8^+$ T cell responses was next examined.

4.3.1.2.2 Effect of hemagglutinin from *Influenza* virus

Many enveloped viruses, including *Influenza* virus, *Herpes simplex* virus (HSV) and HIV, display high-mannose glycoproteins (Schwarz *et al.*, 1977; Basak *et al.*, 1981; Mizuochi *et al.*, 1990; Brunetti *et al.*, 1994; Bolmstedt *et al.*, 2001). *Influenza* virus hemagglutinin (HA) is a major glycoprotein on the surface of influenza viruses. It mediates attachment and entry of the virus into hosts cells, through interactions with lectin receptors (Wiley *et al.*, 1987; White *et al.*, 2005). HA may be involved in triggering the production of IFN-α/β (Miller *et al.*, 2003). Therefore, given *Influenza* HA structure and connection with key elements of the innate immune system, the adjuvant potential of HA was investigated. HA structure varies depending on the virus strain and which cell type *Influenza* was grown in, and egg-grown HA3 viruses were found to interact better with collectins and to induce higher levels of IFN-α than HA1 viruses for example (Reading *et al.*, 1997; Miller *et al.*, 2003). Egg-grown *Influenza* X:31 (H3N2) was thus chosen as a source of HA to study its capacity to generate antigen-specific $CD8^+$ T cell responses.

HA was separated from sucrose gradient-purified *Influenza* X:31 A/Aichi/68 (H3N2) using enzymatic digestion with bromelain, followed by ultracentrifugation (see 2.3.4.3). The bromelain-released HA (B-HA) preparation was characterised by SDS-PAGE in non-reducing conditions (Figure 4.5A). B-HA migration profile showed a band for bromelain-released HA monomer (approximately 80 kDa) (Braakman *et al.*, 1991; Epand *et al.*, 2002) and a band showing carried-over bromelain.

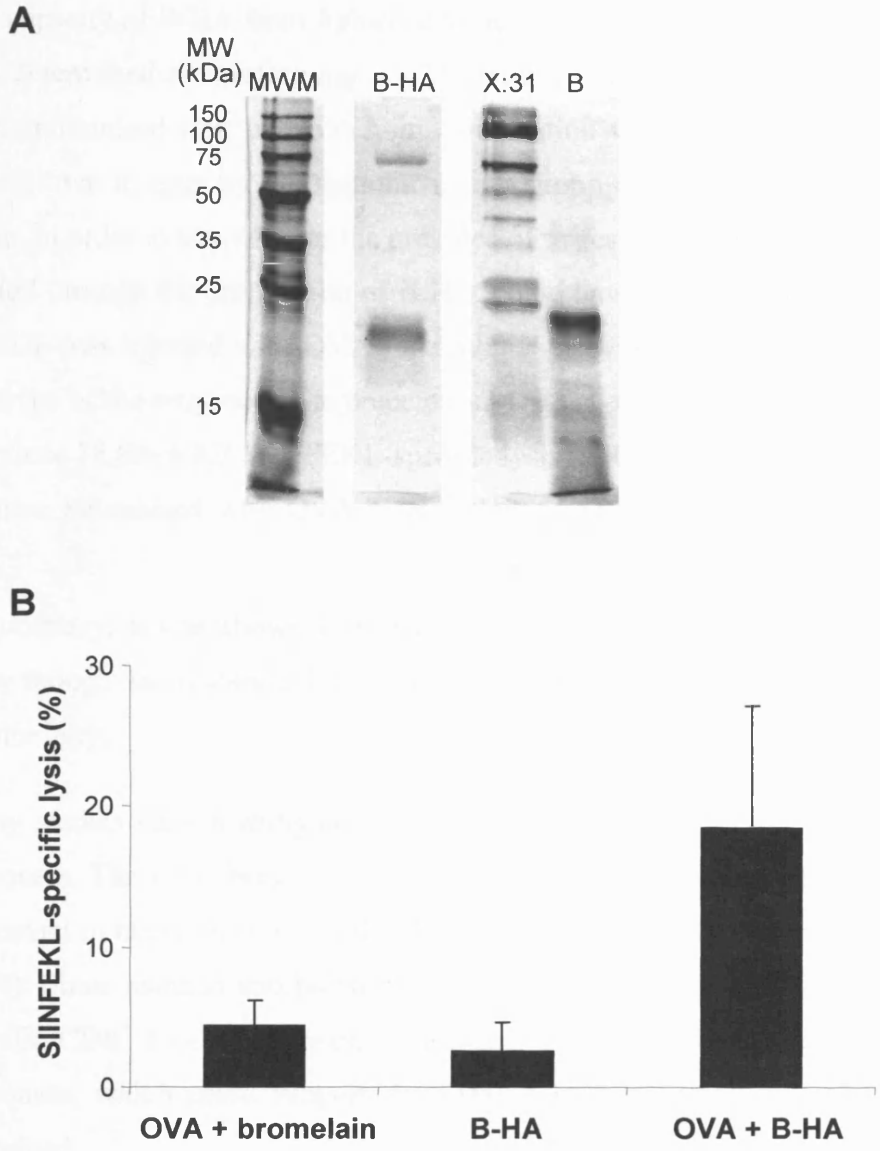

Figure 4.5. The effect of a high-mannose viral protein on induction of cross-priming against OVA. C57Bl/6 mice were injected i.m. with OVA (500 μg) in combination with NaCl-inactivated bromelain (20μg), bromelain-released heamagglutinin (B-HA) from Influenza X:31 A/AICHI/68 (20μg) or OVA (500μg) in combination with B-HA (20μg). (**A**) SDS-PAGE characterisation of bromelain-released HA (B-HA). B-HA was visualised by silver staining after electrophoretic migration in a 12% SDS-polyacrylamide gel, under non-reducing conditions, alongside Influenza virus X:31 (X:31) it was purified from, bromelain (B), the enzyme used to separate HA from X:31, and molecular weight markers (MWM). (**B**) Nine days after immunisation, SIINFEKL-specific $CD8^+$ T cell cytotoxic activity was determined by *in vivo* CTL assay. Results are expressed as percentage of SIINFEKL-specific lysis. Data are represented by mean ± SD for three mice per group. * $p<0.05$ versus control by one-way ANOVA test.

The capacity of B-HA from *Influenza* to induce OVA-specific $CD8^+$ T cell responses was determined by performing a CTL assay *in vivo* (Figure 4.5B). C57Bl/6 mice were immunised i.m. with OVA in combination with B-HA. Since *Influenza* had been grown in eggs by the manufacturer, a group of mice was injected with B-HA alone, in order to test whether the presence of traces of OVA, which might have been carried through the preparation of B-HA, could have an effect in the assay. A group of mice was injected with OVA in combination with bromelain, to account for the presence of the enzyme in the preparation. Data showed that OVA + B-HA was able to induce 18.6% ± 8.7 SIINFEKL-specific lysis ($p=0.043$), while lysis was negligible in mice immunised with OVA + bromelain (4.4% ± 1.8) or B-HA alone (2.5% ± 2.2).

In summary, it was shown that HA, a high-mannose viral glycoprotein, was able, even though to moderate levels, to elicit antigen-specific $CD8^+$ T cell-mediated cytotoxicity.

Many studies have investigated the relevance of $CD4^+$ T cell help in $CD8^+$ T cell responses. There has been a strong consensus that $CD4^+$ T cell responses are indeed important in many aspects of $CD8^+$ T cell responses (Rocha *et al.*, 2004; Smith *et al.*, 2004). Since mannan and polymannose LPS Kp K55 were shown to elicit antigen-specific $CD8^+$ T cell responses, their capacity to induce OVA-specific $CD4^+$ T cell responses, which could support the $CD8^+$ T cell responses observed, was in turn examined.

4.3.2 Antigen-specific $CD4^+$ T cell responses

The effect of mannan, polymannose LPS Kp K55 and rough LPS Kp K52 on expansion of OVA-specific $CD4^+$ T cells, and on the expression profile of cytokines from T-helper cells, was studied.

4.3.2.1 Effect of high-mannose structures on proliferation of antigen-specific $CD4^+$ T cells

OVA-specific $CD4^+$ T cell proliferation was characterised using a model of adoptive transfer of cells from OT II RAG-deficient mice. OT II $RAG^{-/-}$ mice ($CD45.2^+$) have all $CD4^+$ T cells expressing a TCR specific for the MHC II $I\text{-}A^b$ restricted $OVA_{(323\text{-}339)}$ peptide. At the time of immunisation with OVA and polymannose structures,

C57Bl/6 mice ($CD45.1^+$) were also injected with OVA-specific OT II cells. Expansion of the OVA-specific $CD4^+$ T cell population was measured using FACS analysis, after staining cells from recipient mice with both anti-CD4 and anti-CD45.2.

Accordingly, C57Bl/6 ($CD45.1^+$) mice were immunised subcutaneously with OVA alone, or OVA in combination with mannan, LPS Kp K55, or LPS Kp K52. The mice were also injected intravenously with OT II lymph node cells, composed of over 85% $CD4^+$ T cells. Ten days after immunisation, draining inguinal lymph nodes were collected and single cell suspensions were prepared. Cells were labelled with fluorescent anti-CD4 and anti-CD45.2 and their frequency was measured using FACS analysis; representative FACS analysis dot plots are shown in Figure 4.6A. Numbers of OVA-specific $CD4^+$ T cells per draining lymph node (dLN) were calculated from the frequency of $CD45.2^+$ $CD4^+$ cells and dLN cell counts (Figure 4.6B). At day 10, OT II $CD4^+$ T cells, in mice injected with OVA alone, were hardly detectable, probably because the peak of proliferation occurred in the first few days and the population contracted then on. The effect of adjuvants on antigen-specific $CD4^+$ T cells was examined at day 10, thereby after the expected peak in proliferation, because LN cells were also to be restimulated *in vitro* to measure cytokine production, and $CD4^+$ T cells may be killed by restimulation during their early activation phase. In mice immunised with OVA + mannan, the average number of OVA-specific $CD4^+$ T cells increased 9-fold compared to numbers in control mice. In mice immunised with LPS Kp K55 and LPS Kp K52, the OVA-specific $CD4^+$ T cell population expanded more than 10-fold, to 3.2×10^4 cells $\pm$ 2.1×10^4 and 1.1×10^4 cells $\pm$ 0.7×10^4, respectively, compared to 0.08×10^4 cells $\pm$ 0.06×10^4 in control mice.

Mannan and both LPS Kp K55 and LPS Kp K52 were thus shown to enhance proliferation/survival of OVA-specific $CD4^+$ T cells *in vivo*.

The pattern of cytokine expression of these OVA-specific $CD4^+$ T cells was next studied *ex vivo*, after restimulation *in vitro* with OVA peptide.

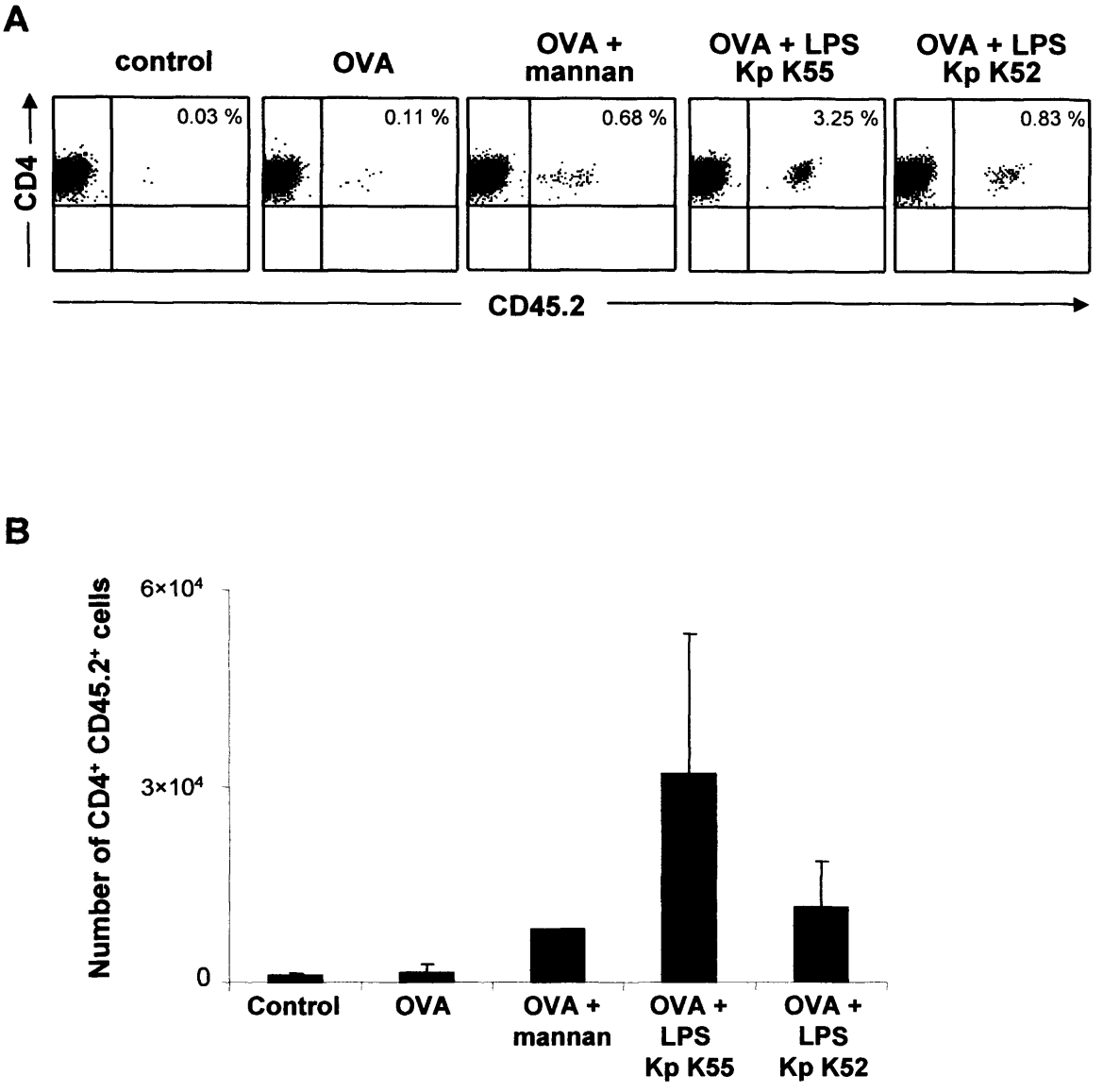

Figure 4.6. The effect of mannan and polymannose lipopolysaccharide on proliferation of antigen-specific CD4$^+$ T cell. C57Bl/6 (CD45.1$^+$) mice were injected s.c. with 500 μg OVA alone or OVA in combination with mannan from *S. cerevisiae* (2.8 mg), LPS from *K. pneumoniae* O3:K55 (Kp K55) (10 μg) or LPS from *K. pneumoniae* K52 (Kp K52) (10 μg). The mice were also injected i.v. with 1.5×10^6 cells prepared from lymph nodes of OT II (CD45.2$^+$) mice. OVA-specific CD4$^+$ T cell proliferation was assessed ten days after immunisation by counting cells recovered from the draining lymph node and determining the proportion of OT II cells using flow cytometry. (A, B) Ten days after immunisation, cells from the draining lymph node were stained with anti-mouse CD4 and anti-mouse CD45.2 antibodies. (A) Representative FACS dot plots of CD45.2 staining. The value in the right corner of each panel represents the percentage of OT II cells (CD45.2$^+$) among CD4$^+$ T cells. (B) Results are expressed as absolute number of OVA-specific CD4$^+$ OT II cells recovered from the draining lymph node (dLN). Data are represented by mean ± SD.

4.3.2.2 Cytokine production by $CD4^+$ T cells primed in the presence of high-mannose molecules

$CD4^+$ T cells, purified from lymph nodes ten days after immunisation, were cultured with syngeneic stimulator cells, in the presence or absence of 5 μM $OVA_{(323-339)}$ peptide (see 2.7.2). Culture supernatants were collected 72 h later and assayed for the presence of prototypic Th0 (IL-2), Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines (Figure 4.7). It is to note that no cytokines were detectable in wells where stimulator cells were cultured on their own, both in the presence or absence of OVA peptide. The cytokine array data were expressed as cytokine concentration produced per 10^5 OT II $CD4^+$ T cells. Antigen-specific $CD4^+$ T cells from both control and OVA-injected mice exclusively produced IL-2. In contrast, mannan, LPS Kp K55 and LPS Kp K52 induced the production of IFN-γ, in addition to IL-2. Mannan stimulated predominantly the production of IL-2, while LPS Kp K55 induced more IFN-γ than IL-2. LPS Kp K52 induced relatively equivalent levels of IL-2 and IFN-γ. Mannan, LPS Kp K55 and LPS Kp K52 elicited no, or negligible, production of IL-4 and IL-5. Thus, data showed that mannan, LPS Kp K55 and LPS Kp K52 induced similar mixed Th0/Th1 cytokine expression profiles from OVA-specific $CD4^+$ T cells, though cytokine levels varied.

In summary, mannan and both polymannose-LPS Kp K55 and rough LPS Kp K52 were able to induce antigen-specific $CD4^+$ T cell responses, as they stimulated proliferation of OVA-specific $CD4^+$ T cells and instructed the production of Th0 and Th1 cytokines from those cells, specifically in response to OVA.

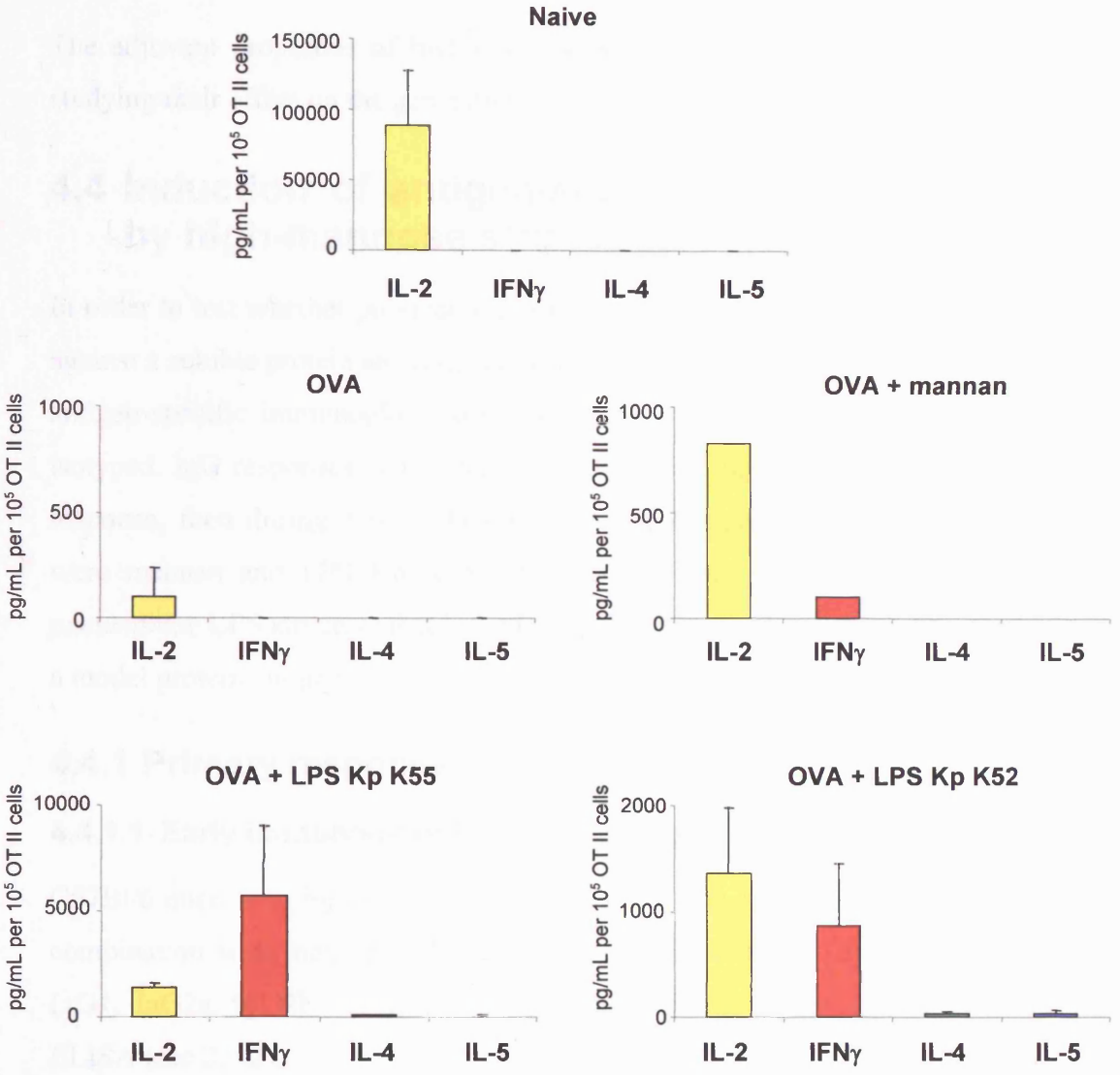

Figure 4.7. Cytokine production from antigen-specific CD4$^+$ T cells primed in the presence of mannan and polymannose lipopolysaccharide. C57Bl/6 (CD45.1$^+$) mice were injected s.c. with 500 µg OVA alone or OVA in combination with mannan from *S. cerevisiae* (2.8 mg), LPS from *K. pneumoniae* O3:K55 (Kp K55) (10 µg) or LPS from *K. pneumoniae* K52 (Kp K52) (10 µg). The mice were also injected i.v. with 1.5×10^6 lymph nodes cells from OT II (CD45.2$^+$) mice. Ten days after immunisation, CD4$^+$ T cells were purified from the draining lymph node and seeded at 1×10^6 cells / mL with 5×10^6 / mL syngeneic T-depleted splenocytes, in the presence or absence of 5 µM $OVA_{(323-339)}$ peptide. Culture supernatants were collected after 72 h incubation, and cytokine concentration was determined using flow cytometry array (mouse Th1/Th2 cytometric bead array; BD Biosciences). Results are expressed as concentration of cytokine produced per 10^5 CD4$^+$ OT II cells; concentration of cytokine produced in the absence of OVA peptide was subtracted. Data are represented by mean ± SD.

The adjuvant properties of high-mannose structures were characterised further by studying their effect on the generation of antigen-specific B-cell responses.

4.4 Induction of antigen-specific humoral responses by high-mannose structures

In order to test whether polymannose molecules are able to elicit humoral responses against a soluble protein antigen, and to evaluate the quality of this response, titres of antigen-specific immunoglobulin G were measured in serum, and antibodies were isotyped. IgG responses were characterised first during an early and a late primary response, then during a secondary response. The high-mannose structures studied were mannan and LPS Kp K55. LPS Kp K52 was included, to represent a *K. pneumoniae* LPS structure that lacked the polymannose O-antigen. CGG was used as a model protein antigen.

4.4.1 Primary response

4.4.1.1 Early immunoglobulin G responses

C57Bl/6 mice were injected once intramuscularly (i.m.) with CGG alone, or CGG in combination with mannan, LPS Kp K55, or LPS Kp K52. Titres of CGG-specific IgG1, IgG2a, IgG2b and IgG3 were measured in the serum ten days later, using ELISA (see 2.5.2).

CGG induced weak IgG responses, which consisted mainly of IgG1 (37,333 ± 19,956) and very little, if any, IgG2a (50 ± 0), IgG2b (467 ± 249) or IgG3 (250 ± 0). Co-administration of mannan, LPS Kp K55 or LPS Kp K52 increased CGG-specific responses, with the production of all IgG isotypes being enhanced (Figure 4.8). Results are expressed as fold increase in antibody endpoint titre compared to titres induced by CGG on its own.

Titres of anti-CGG IgG1 were higher in mice immunised with CGG + mannan (11.4-fold ± 3.2, p=0.011), CGG + LPS Kp K55 (22.9-fold ± 6.5, p=0.009) and CGG + LPS Kp K52 (13.7-fold ± 0.0, p=0.000), compared to IgG1 titres in mice injected with CGG alone.

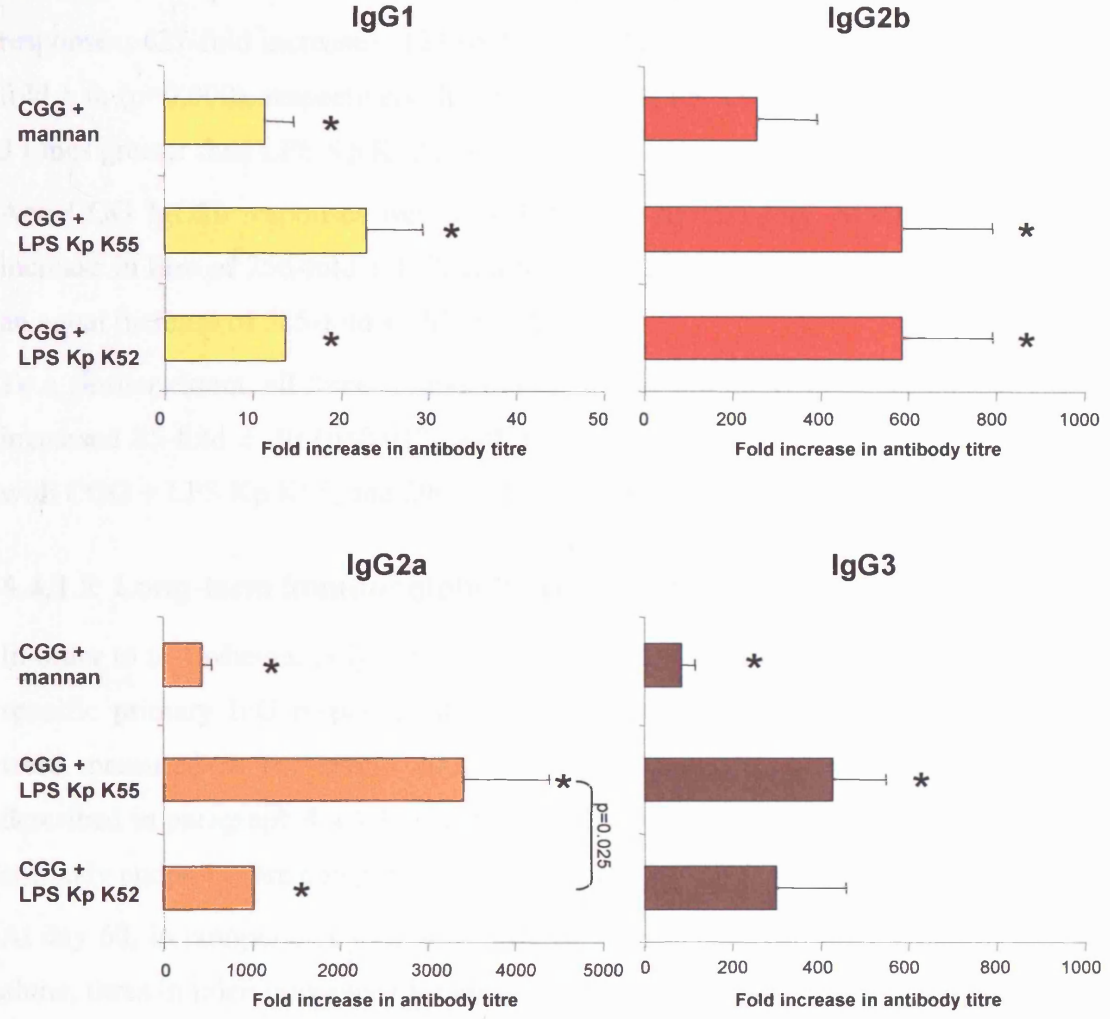

Figure 4.8. The effect of mannan and polymannose lipopolysaccharide on primary antigen-specific antibody responses. C57Bl/6 mice were injected i.m. with 100 μg chicken γ-globulin (CGG) alone or CGG in combination with mannan from *S. cerevisiae* (2.8 mg), LPS from *K. pneumoniae* O3:K55 (Kp K55) (10 μg) or LPS from *K. pneumoniae* K52 (Kp K52) (10 μg). Ten days after immunisation, serum samples were collected and titres of CGG-specific IgG1, IgG2b, IgG2a and IgG3 were measured using ELISA. Results are expressed as fold-increase in CGG-specific antibody titre compared with titres in mice immunised with CGG alone. Data are represented by mean ± SD for three mice per group. * $p<0.05$ versus CGG immunisation group, by one-way ANOVA test.

Mannan, LPS Kp K55 and LPS Kp K52 induced extremely potent anti-CGG IgG2a responses: 427-fold increase ± 121 (p=0.008), 3413-fold ± 965 (p=0.007), and 1024-fold ± 0, (p=0.000), respectively. It is to note that LPS Kp K55 generated a response 3 times greater than LPS Kp K52 (p=0.025).

Anti-CGG IgG2b responses were also enhanced significantly. Mannan induced an increase in titre of 256-fold ± 137, and both LPS Kp K55 and LPS Kp K52 induced an equal increase of 585-fold ± 207 (p=0.016).

To a similar extent, all three compounds enhanced anti-CGG IgG3 responses. Titres increased 85-fold ± 30 (p=0.017) with CGG + mannan, 427-fold ± 121 (p=0.008) with CGG + LPS Kp K55, and 299-fold ± 160 with CGG + LPS Kp K52.

4.4.1.2 Long-term immunoglobulin G responses

In order to test whether polymannose molecules could generate a long-lasting CGG-specific primary IgG response, titres of anti-CGG IgG1, IgG2a, IgG2b and IgG3 were measured in the serum sixty days after the single immunisation protocol described in paragraph 4.4.1.1 (Figure 4.9). Results are expressed as fold increase in antibody endpoint titre compared to titres induced by CGG injected alone.

At day 60, in comparison with anti-CGG IgG1 titres in mice immunised with CGG alone, titres in mice previously immunised with CGG + LPS Kp K55 were higher by 24.0-fold ± 11.3 (p=0.045). Mannan and LPS Kp K52 induced IgG1 titres that were only higher by 7.3-fold ± 6.2 and 5.3-fold ± 1.9 (p=0.031), respectively.

Anti-CGG IgG2a titres, in mice previously immunised with the CGG + adjuvant were also higher than titres in mice injected with CGG alone. Fold-increases in antibody titres in mice immunised with mannan, LPS Kp K55 or LPS Kp K52 were 34.7-fold ± 22.9, 512-fold ± 0 (p=0.000), and 213 ± 60 (p=0.008), respectively. The response induced by LPS Kp K55 was more than twice greater than the response induced by LPS Kp K52 (p=0.002).

In mice previously immunised with CGG + mannan, titres of anti-CGG IgG2b at day 60 were 32-fold ± 0 (p=0.000) higher than titres in mice injected with CGG alone. Fold-increases in CGG-specific IgG2b titres induced by CGG + LPS Kp K55 and CGG + LPS Kp K52 were 149 ± 80 and 85 ± 30 (p=0.017), respectively.

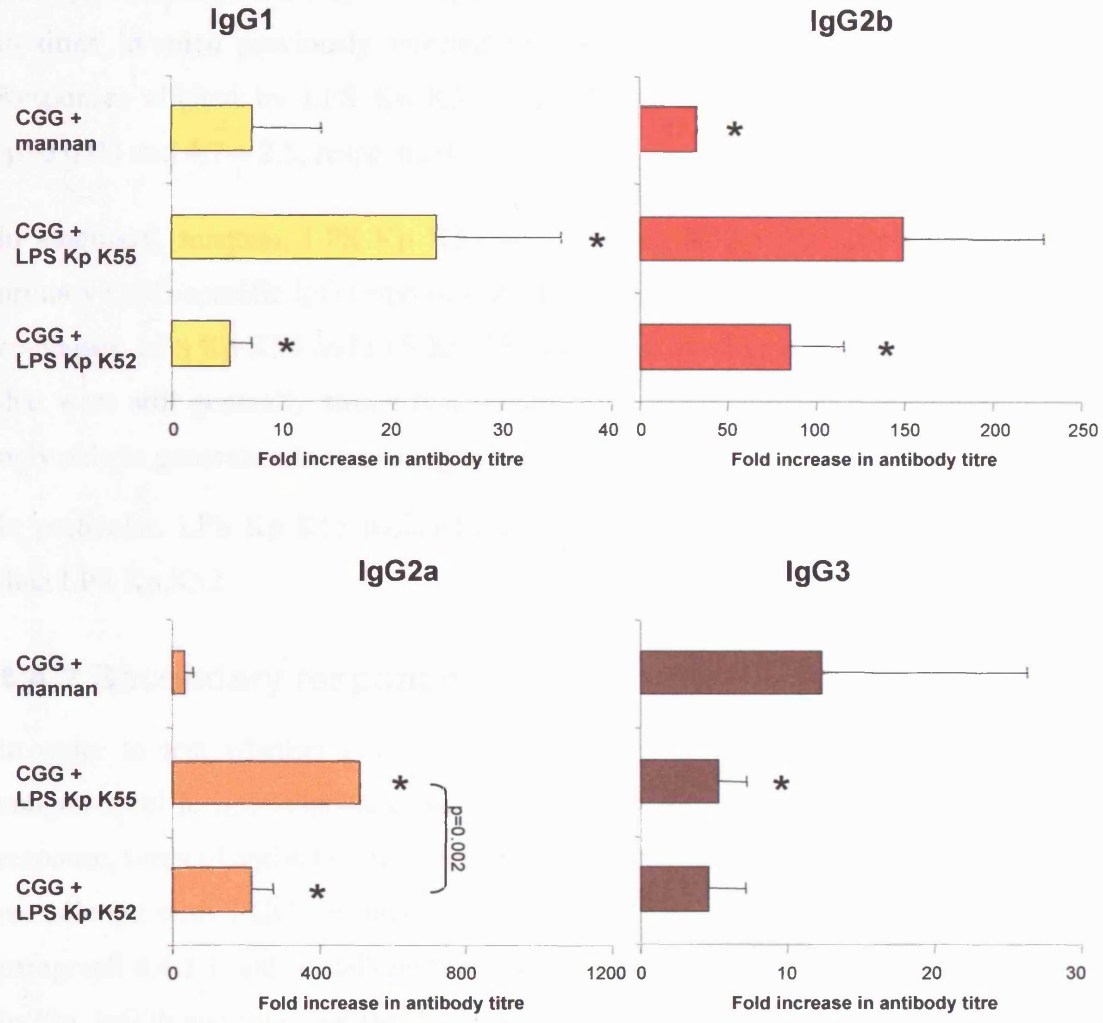

Figure 4.9. The effect of mannan and polymannose lipopolysaccharide on long-term antigen-specific antibody responses. C57Bl/6 mice were injected i.m. with 100 μg chicken γ-globulin (CGG) alone or CGG in combination with mannan from *S. cerevisiae* (2.8 mg), LPS from *K. pneumoniae* O3:K55 (Kp K55) (10 μg) or LPS from *K. pneumoniae* K52 (Kp K52) (10 μg). Sixty days after immunisation, serum samples were collected and titres of CGG-specific IgG1, IgG2b, IgG2a and IgG3 were measured using ELISA. Results are expressed as fold-increase in CGG-specific antibody titre compared with titres in mice immunised with CGG alone. Data are represented by mean ± SD for three mice per group. * $p<0.05$ versus CGG immunisation group, by one-way ANOVA test.

At day 60, titres of CGG-specific IgG3 induced by mannan were in fact comparable to titres in mice previously injected with CGG alone (12.3-fold increase ± 14) Responses elicited by LPS Kp K55 and LPS Kp K52 were low: 5.3-fold ± 1.9 ($p=0.031$) and 4.7 ± 2.5, respectively.

In summary, mannan, LPS Kp K55 and LPS Kp K52 were all able to enhance primary CGG-specific IgG responses. All three compounds induced very potent early responses. LPS Kp K55 and LPS Kp K52 were able to elicit anti-CGG IgG responses that were still generally strong two months after immunisation, while mannan was only able to generate a long-term IgG2b response.

In particular, LPS Kp K55 induced a better CGG-specific IgG2a primary response than LPS Kp K52.

4.4.2 Secondary response

In order to test whether polymannose molecules were able to generate memory antigen-specific IgG responses, while minimising the contribution of a new primary response, titres of anti-CGG IgG isotypes were measured in the serum five days after rechallenge with CGG, in mice, which had been immunised once as described in paragraph 4.4.1.1 and rechallenged at day 61. Endpoint titres of CGG-specific IgG1, IgG2a, IgG2b and IgG3, on the day before and 5 days after rechallenge, are shown in Figure 4.10.

Titres of CGG-specific IgG, of all isotypes, induced by CGG on its own were identical before and after challenge.

In mice immunised with OVA + mannan, anti-CGG IgG1 titres more than doubled after rechallenge, from 58,667 ± 49,459 to 149,333 ± 78,822. In mice immunised with LPS Kp K55 ($p=0.002$) or LPS Kp K52, titres increased more than ten times after challenge, though LPS Kp K55 induced a greater secondary response than LPS Kp K52 ($p=0.006$).

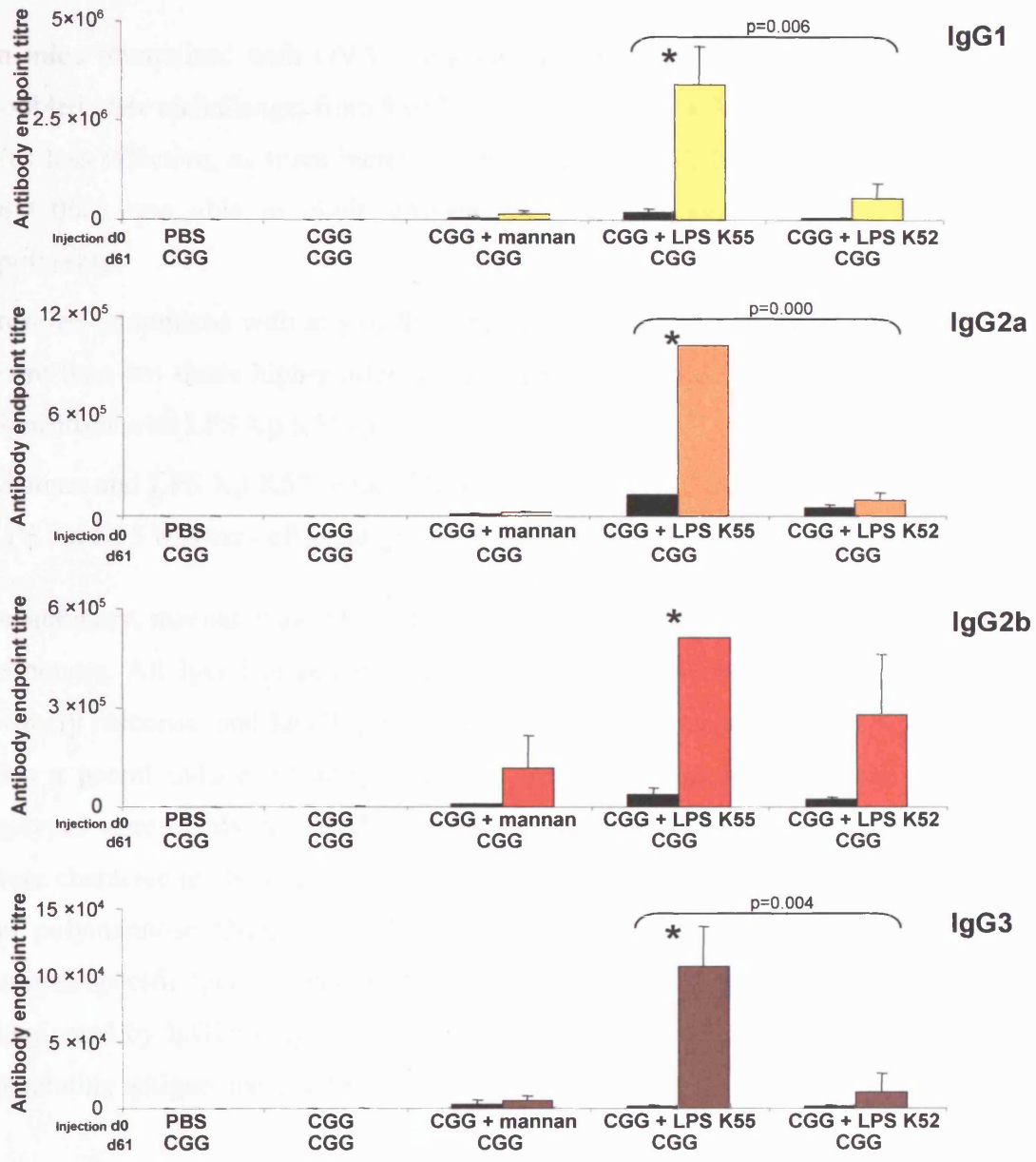

Figure 4.10. The effect of mannan and polymannose lipopolysaccharide on secondary antigen-specific antibody responses. C57Bl/6 mice were injected i.m. with PBS or 100 μg chicken γ-globulin (CGG) alone or CGG in combination with mannan from *S. cerevisiae* (2.8 mg), LPS from *K. pneumoniae* O3:K55 (K55) (10 μg) or LPS from *K. pneumoniae* K52 (K52) (10 μg). Sixty-one days after immunisation, mice were rechallenged i.p. with 100 μg CGG. Five days later, serum samples were collected and titres of CGG-specific IgG1, IgG2a, IgG2b and IgG3 before rechallenge (■) and after rechallenge (▮▮▮▮) were measured using ELISA. Results are expressed as CGG-specific antibody endpoint titre. Data are represented by mean ± SD for three mice per group. * $p<0.05$ versus CGG immunisation groups (before and after challenge), by ANOVA General Linear Model test.

In mice immunised with OVA + mannan, anti-CGG IgG2a titres also more than doubled after rechallenge, from 8,667 ± 5.734 to 21,333 ± 7,542, while LPS Kp K52 was less effective, as titres increased less than two-fold. In contrast, LPS Kp K55 ($p=0.000$) was able to elicit stronger secondary responses than LPS Kp K52 ($p=0.000$).

In mice immunised with any of the three compounds, anti CGG-IgG2b titres were more than ten times higher after rechallenge. IgG2b secondary response was most significant with LPS Kp K55 ($p=0.000$).

Mannan and LPS Kp K52 induced little secondary anti-CGG IgG3 responses, while LPS Kp K55 was very efficient ($p=0.001$), and more so than LPS Kp K52 ($p=0.004$).

In summary, mannan was able to induce primary and secondary antigen-specific IgG responses. All IgG isotypes were produced, but IgG2a and IgG2b dominated the primary response, and IgG2b prevailed during memory responses. LPS Kp K55 was also a potent inducer of antigen-specific IgG responses. IgG2a, IgG2b and IgG3 isotypes were highly produced during the primary response, and memory responses were characterised by even greater levels of IgG2a and IgG2b. Although possessing no polymannose O-antigen, LPS Kp K52 elicited the same profile of primary antigen-specific IgG response as LPS Kp K55. In contrast, secondary responses were dominated by IgG2b only. Overall, LPS Kp K55 was significantly more efficient in generating antigen-specific IgG2a responses.

4.5 Conclusions and discussion

Carbohydrates fulfil important biological functions and are indispensable to many organisms. Immune systems have evolved to recognise and react adequately to carbohydrate structural constitutents, from as diverse as lipopolysaccharides from bacteria, viral glycoproteins, and yeast β-glucan and mannan. Particular immunostimulatory activities displayed by some carbohydrates have led to vaccine development studies investigating the use of polysaccharides as adjuvants, capable of safely enhancing immune responses against co-administered antigens. The aim of the work reported here was to examine the potential of mannan and other microbial high-mannose molecules as stimulators of cellular and humoral immune responses against soluble protein antigens.

As an initial approach, mannan was conjugated under oxidising conditions to a model protein antigen, OVA. This procedure, applied to tumour antigen MUC1, had been shown to generate immunogenic conjugates able to induce antigen-specific $CD8^+$ T cell responses in mice (Apostolopoulos *et al.*, 1995). The OVA model studied here however showed a different outcome. Indeed, OVA-oxMan conjugates were not able to induce cross-priming against OVA, at least during a primary response. Aldehydes, produced by oxidation of mannan, were found to be essential for the adjuvant functions of MUC1-oxMan conjugates (Apostolopoulos *et al.*, 2000). Aldehydes had previously been proven to have immunostimulatory and adjuvant properties, whether conjugated to a target antigen or simply co-administered (Soltysik *et al.*, 1995; Cross *et al.*, 2001). It was therefore hypothesised that oxidised mannan and OVA might require to interact with different innate cell types, independently, for adjuvant mechanisms to take place. OxMan, co-injected with OVA, was not able either to induce cross-priming against OVA, despite presenting similar amounts of aldehydes as described by (Apostolopoulos *et al.*, 2000) (0.27 to 0.47 μmol/mg mannan in the present study, determined colorimetrically with 2,4-dinitrophenylhydrazine). Adjuvant properties of aldehydes, in generating functional antigen-specific $CD8^+$ T cell responses in particular, therefore probably depend on a multiple-immunisation course, in addition to the nature of the target antigen. Indeed, studies relative to MUC1-oxMan describe the effect of conjugates after three injections in mice, and after four to seven injections in primates (Apostolopoulos *et al.*, 1995; Vaughan *et al.*, 1999). Immunisation of mice with three doses of oxMan conjugated to a different antigen, *Listeria monocytogenes* listeriolysin O (LLO), did not result either in antigen-specific CTL responses, whether conjugates were inoculated intranasally or intraperitoneally (Stambas *et al.*, 2002a; Stambas *et al.*, 2002b). OxMan conjugates might represent more promising anti-infectious adjuvants when integrated into modern immunisation protocols, such as prime-boost strategies, and especially those aiming at supporting antibody responses (Stambas *et al.*, 2005).

If the use of oxidised mannan was not a successful approach for generating primary antigen-specific $CD8^+$ T cell responses, free native mannan manifested exciting adjuvant properties. Mannan, co-administered with a protein antigen, consistently stimulated specific adaptive responses. Mannan's adjuvant effect was diversified, as it affected both cellular and humoral adaptive responses. Cellular responses proved

to be functional, since clonal expansion of antigen-specific $CD8^+$ and $CD4^+$ T cells was accompanied by differentiation into effector cells. Indeed, mannan induced functional cross-priming, since antigen-specific $CD8^+$ T cells, generated in the presence of mannan, proliferated and were capable of producing IFN-γ upon restimulation. Effector $CD8^+$ T cells were also armed with granzyme B for cytotoxic killing, and demonstrated their ability to lyse target cells *in vivo*. Antigen-specific $CD4^+$ T cells, generated in the presence of mannan, also demonstrated, upon restimulation, their capacity to become effector cells. Indeed, some antigen-specific $CD4^+$ T cells exhibited a Th1 phenotype, characterised by the secretion of IFN-γ. This indicated that antigen-specific $CD4^+$ T cells generated in the presence of mannan had the potential to enhance cellular responses, through induction of cytokine production for example (Kipps *et al.*, 1985; Gracie *et al.*, 1996). This interpretation was supported, indirectly, by the presence of serum antigen-specific immunoglobulin G of various isotypes. Indeed, it has been established that, in order to respond to protein antigens and undergo appropriate antibody class switching, B cells require help from antigen-specific $CD4^+$ T cells, through CD40/CD40L interactions. IFN-γ produced by $CD4^+$ T cells, whilst not indispensable, favours B cell responses that can also support cellular responses. This includes, in mice, the production of IgG2a antibodies, which are characterised by their strong capacity to induce antibody-mediated cellular cytotoxicity (ADCC) by NK cells and macrophages, through FcγRI and FcγRIII (Gavin *et al.*, 1998; Barnes *et al.*, 2002b; Radaev *et al.*, 2002). In fact, mannan enhanced production of antigen-specific IgG2a, which may correlate with the capacity of antigen-specific $CD4^+$ T cells, generated in the presence of mannan, to secrete IFN-γ. Mannan also stimulated the production of antigen-specific IgG of other isotypes (IgG1, IgG2b, IgG3). The antibody isotypes enhanced by mannan have a variety of functions, in terms of neutralisation and opsonisation. Opsonization allows both FcR targeting on phagocytes, and complement fixation. Mannan has been shown for example to promote the secretion of neutralising anti-rotavirus antibodies (Franklin *et al.*, 2005). IgG1 and IgG2a antibodies in particular are efficient activators of the complement classical pathway, which helps antigen removal. They can also induce complement-mediated cytotoxicity (CMC) (Klaus *et al.*, 1979; Herlyn *et al.*, 1985).

With appropriate help from $CD4^+$ Th cells, including CD40L/CD40 ligation, and then survival signals in the bone marrow, antigen-specific B cells can differentiate and survive as long-lived antibody-secreting plasma cells (McHeyzer-Williams *et al.*, 2005). Long-term antigen-specific antibody responses were enhanced in mice immunised once with antigen + mannan, indicating that during the primary response mannan provided signals that were integrated by antigen-specific B cells, and/or antigen-specific $CD4^+$ T cells, and/or follicular DC. As a result, some B cells differentiated into long-lived plasma cells, which were still producing antigen-specific antibodies 60 days after initial priming, without second antigenic or adjuvant stimulation. Some B cells can also differentiate into memory B cells, which do not secrete antibodies, but are rapidly able to if their BCR is activated. Five days after rechallenge with antigen only, antigen-specific antibody responses were enhanced, compared to responses before rechallenge, in mice that had been immunised once, 61 days earlier, with antigen and mannan. Antibodies could not have been produced within five days by newly primed B cells, because B cell differentiation into antibody-secreting plasma cells would require at least seven days. This indicated that during the primary response, mannan was able to stimulate the instruction of B cell differentiation into memory cells. Mannan, co-administered with a protein antigen, promoted the generation of long-lived and memory antigen-specific B cells characterised by two IgG isotypes in particular: IgG2b and IgG2a, which are isotypes that contribute to immune mechanisms most efficient at eliminating intracellular pathogens.

Mannan is a complex glycan, and its adjuvant effects may be related to its composition. Mannan is a yeast cell wall polysaccharide, composed of mannose polymers, such as the linear backbone chain made of α-1,6 mannose units, and mannose oligomers, such as the linear or branched side chains of α-1,2 and α-1,3 mannose units. Other organisms, including some viruses (Deom *et al.*, 1986; Kohl *et al.*, 2004; Wang *et al.*, 2004), bacteria (Prehm *et al.*, 1976; Paeng *et al.*, 1996; Tian *et al.*, 2000) and plants (Gauntt *et al.*, 2000) bear high-mannose molecules. While polysaccharides in general were suspected early on of having immunomodulatory activities (Suzuki *et al.*, 1969a; Suzuki *et al.*, 1969b; Lee *et al.*, 2001), only a few studies have reported on adjuvant properties of mannan (Domer *et al.*, 1986; Franklin *et al.*, 2005) or other polymannose molecules (Ohta *et al.*, 1982; Kido *et al.*, 1985a).

Results presented here demonstrated a novel and exciting feature of mannan: its ability to induce antigen-specific $CD8^+$ T cell responses. Endotoxin contamination is often suspected to account for the activity of purified microbial components. This possibility was considered. The endotoxin content of mannan (15 ng/mg) was measured by the *Limulus* amoebocyte assay (see 2.3.4.2). However, this measure can be inaccurate, since β–(1,3) glucans, which may have been carried over in small amounts during mannan precipitation, activate the clotting enzymes used in the assay and therefore bias the readings of endotoxin activity (Ohno *et al.*, 1990). An endotoxin-specific *Limulus* test should have been used. The possible adjuvant activity of β-glucan contaminants was excluded, as up to 500 μg of purified β-(1,3) glucans were not able to induce cross-priming against soluble OVA. In addition, periodate oxidisation, which is often used to test the specific activity of carbohydrates, was shown to abrogate the adjuvant effect of free mannan (see Figure 4.2), while lipid A structures from possible endotoxin contaminants would have been resistant to periodate. Furthermore, the activity of mannan (Tada *et al.*, 2002), and other polysaccharides (Flo *et al.*, 2002; Saito *et al.*, 2003), was previously shown to be resistant to polymyxin B, which binds to lipid A and inhibits its activity. Ideally, studies would be performed using compounds synthesised in a controlled environment. However, synthesis of complex carbohydrates is only in early stages of development.

Influenza HA and LPS from *K. pneumoniae* K55 both contain immunologically relevant polymannose structures (Roberts *et al.*, 1993; Reading *et al.*, 1997; Zhao *et al.*, 2002) and since purification methods are well established for these two molecules (Bonnafous *et al.*, 2000; Zamze *et al.*, 2002), HA and LPS Kp K55 were chosen to examine whether high-mannose compounds also manifested the ability to induce cross-priming. HA and LPS Kp K55 were indeed shown to induce antigen-specific $CD8^+$ T cell responses. HA and LPS Kp K55 are admittedly hybrid molecules, as HA is a glycoprotein and LPS Kp K55 a glycolipid. Therefore, their adjuvant effect cannot be irrefutably attributed to their high-mannose fraction, although previous studies have illustrated the prominent contribution of the polysaccharide moiety in the immunogenicity and/or adjuvanticity of HA and LPS Kp K55. Indeed, sodium periodate oxidises adjacent hydroxyls on sugar rings, such as found in HA, and strong periodate treatment of influenza virus was shown to

result in a great reduction of IFN-α/β induction, which was imputed to HA loss of conformation (Miller *et al.*, 2003). The present study did not examine the precise contribution of the high-mannose structure to the adjuvant effect of HA. One way of addressing this in the future would be to destroy or remove HA high-mannose fraction, by periodate treatment or mannosidases digestion. Another way would be to compare a normally glycosylated HA with HA naturally lacking high-mannose (Nakamura *et al.*, 1979; Reading *et al.*, 2000).

LPS Kp K55 marked adjuvanticity has been shown to depend on its polymannose fraction. LPS Kp K55 lipid A, separated from the polymannose O-antigen by hydrolysis, displayed a great loss of adjuvant activity (Kato *et al.*, 1985), and comparative studies with an O-antigen deficient mutant confirmed that the polymannose moiety was indispensable to LPS adjuvant effect in inducing delayed-type hypersensitivity to OVA, and in augmenting antibody responses to bovine γ-globulin (Ohta *et al.*, 1987). LPS from *E. coli* K-12 is a weak enhancer of antigen-specific antibody responses; however when *E. coli* K-12 was transfected with *rfb* genes capable of synthesizing the polymannose O-antigen, its LPS adjuvant activity was significantly increased (Paeng *et al.*, 1996). In contrast to polymannose O-antigen (*Klebsiella* serotype O3) in LPS Kp K55, O-antigens with a galactose structure in LPS K1:O1 and K3:O2 did not correlate with an enhanced adjuvant activity (Jones, 2004). In the present study, LPS Kp K52, a rough LPS from *K pneumonia*, was used in comparison with LPS Kp K55, in an attempt to determine the contribution of the polymannose O-antigen to adjuvanticity, although a O-antigen deficient mutant of LPS Kp K55 would have been a better control. If no significant difference could be established between the polymannose LPS and the rough LPS in their capacity to induce cross-priming, the polymannose LPS displayed a stronger activity in enhancing antigen-specific antibody responses. More precisely, LPS Kp K55 was able to more strongly promote antigen-specific IgG2a responses, which play an important role in protective responses, since IgG2a antibodies are involved in assisting and inducing cell-mediated cytotoxicity (CMC and ADCC). Although LPS Kp K52 and LPS Kp K55 both enhanced antigen-specific $CD4^+$ T cell proliferation and promoted a similar $CD4^+$ T helper cell differentiation pattern, LPS Kp K55 was generally over five times more potent than the rough LPS, which is consistent with results reported by (Ohta *et al.*, 1987) and (Paeng *et al.*, 1996). LPS Kp K55 greater

adjuvant effect on augmenting antibody responses, regarding IgG isotype switching especially, would also be consistent with greater adjuvanticity on $CD4^+$ T cell responses.

Overall, LPS Kp K55 polymannose O-antigen appears to increase the adjuvant effect of the lipid A/core moiety on augmenting antigen-specific $CD4^+$ T cell responses, and on augmenting a particular type of humoral responses that may be associated with $CD8^+$ T cell-non-mediated cytotoxicity.

Mannan and polymannose LPS Kp K55 were thus shown to induce antigen-specific $CD8^+$ T cell responses and to enhance antigen-specific $CD4^+$ T cell and antigen-specific B cell responses. These carbohydrates thereby displayed properties that make them potential adjuvants against intracellular pathogens in particular, against which cytotoxic mechanisms are essential. In view of their disparate strength of activity, LPS Kp K55 being more potent than mannan, but considering the known toxicity of lipid A, engineering a molecule that contains the polymannose fraction linked to a less endotoxic lipid A/lipid A analog may constitute a fruitful strategy.

Understanding how mannan and high-mannose molecules trigger the adjuvant activities reported here would be pertinent, in order to try to control and make full use of their properties, for potential prophylactic/therapeutic ends.

As a first step in that direction, while attempting to define a common mechanism by which PAMPs in general induce antigen-specific $CD8^+$ T cell responses against a soluble protein antigen, the way(s) mannan and high-mannose molecules license cross-priming was examined, as reported in the following chapter.

Chapter 5

Mechanisms of induction of cross-priming against a soluble protein antigen by pathogen-associated molecular patterns

5.1 Introduction

Cross-priming refers to the productive activation of $CD8^+$ T cells against an exogenous antigen, meaning an antigen that was not synthesised within the APC it is presented by, but was captured. Similarly to classical $CD8^+$ T cell priming against an endogenous antigen, cross-priming requires that $CD8^+$ T cells interact with APC that not only present the antigen on MHC I, but are also appropriately activated (Bonifaz *et al.*, 2002). Indeed, as presentation of endogenous antigen to $CD8^+$ T cells by resting DC leads to tolerance, cross-presentation under steady state leads to cross-tolerance (Guerder *et al.*, 1992; Kurts *et al.*, 1997).

Activation of DC that allows for cross-priming has been termed licensing (Keene *et al.*, 1982; Lanzavecchia, 1998), and to date two major licensing stimuli have been described. Cognate $CD4^+$ T cell help has been shown to provide essential/sufficient signalling to APC (Bennett *et al.*, 1997), through CD40L/CD40 interactions (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998; Lefrancois *et al.*, 2000), for carrying out both cellular cross-priming and cross-priming against soluble antigens. More recently, IFNα/β was identified as a soluble licensing factor, which acts independently of $CD4^+$ T cell help and CD40/CD40L ligation (Le Bon *et al.*, 2003).

Some microbial products, essentially PAMPs, are detected by the innate immune system, and this triggers specific intracellular signalling pathways that activate the transcription of particular sets of genes, leading to the expression of costimulatory molecules and cytokines. Activation pathways stimulated by PAMPs include the NF-κB pathway (Takeuchi *et al.*, 1999; Hemmi *et al.*, 2000; Alexopoulou *et al.*, 2001) and the IRF pathways (Kawai *et al.*, 2001; Yamamoto *et al.*, 2002b; Yamamoto *et al.*, 2003; Kawai *et al.*, 2004). There is a potential strong link between PRR

signalling and PAMP adjuvant activities, which at present has been made clear for TLR signalling in particular. Enhancement of adaptive humoral responses by CFA, for example, was shown to be mediated by TLR signalling, and also to be dependent on IFNα/β signalling (Le Bon *et al.*, 2001; Schnare *et al.*, 2001). Hence, PRR activation and stimulation of autocrine/paracrine cytokines networks may promote CD40/CD40L expression/ligation and/or IFN-α/β secretion, which in turn may mediate licensing for cross-priming. dsRNA motifs for instance, are recognised by various receptors, including TLR3, PKR, and RNA helicases RIG-1 and mda-5, and can induce IFN-α/β production and expression of costimulatory molecules (Diebold *et al.*, 2003; Hoebe *et al.*, 2003b; Honda *et al.*, 2003; Oshiumi *et al.*, 2003a; Andrejeva *et al.*, 2004; Kato *et al.*, 2005). LPS from *E. coli* (TLR4) and CpG DNA (TLR9) are also able to induce IFN-α/β and costimulatory molecules (Kaisho *et al.*, 2001; Hemmi *et al.*, 2003; Oshiumi *et al.*, 2003b; Zhao *et al.*, 2003; Tabeta *et al.*, 2004).

Having established, in chapters 3 and 4, the ability of various PAMPs to induce cross-priming against a soluble protein antigen, it was of importance to investigate which licensing pathways were involved. The study of licensing mechanisms in response to TLR agonists was focussed on the stimulation of innate cytokines and the role of IFN-α/β in particular, for two reasons. Firstly, the TLR agonists found to induce cross-priming (poly(I:C), LPS and CpG DNA) have all been shown to induce IFN-α/β that are able to mediate stimulation of T cell responses (Tough *et al.*, 1996; Tough *et al.*, 1997; Sun *et al.*, 1998), which may favour adjuvanticity. Secondly, IFN-α/β, which those agonists have the capacity to induce, are able to convert a T-helper cell-dependent cross-priming into a T-helper cell-independent process (Le Bon *et al.*, 2003). This suggests that poly(I:C) and LPS are probably able to induce cross-priming independently of $CD4^+$ T cell, a fact that has already been demonstrated for CpG DNA (Cho *et al.*, 2000; Cho *et al.*, 2002). Induction of cross-priming by particular carbohydrate structures, such as high-mannose carbohydrates, has not been reported before. The contribution of various known cross-priming licensing stimuli, including IFN-α/β signalling since some high-mannose glycoproteins have been shown to induce IFN-α production (Mone *et al.*, 1992; Milone *et al.*, 1998; Miller *et al.*, 2003; Rong *et al.*, 2003), needed to be assessed.

While much progress has been made over the last five years on mapping intracellular signalling cascades that are initiated by TLR, and on linking TLR stimulation to adaptive responses, characterisation of how other PRRs, such as C-type lectins, participate in instructing adaptive immune responses is still in its infancy. Cooperation between TLR and C-type lectins has however been reported (Perera *et al.*, 2001; Brown *et al.*, 2003; Gantner *et al.*, 2003a; Nagaoka *et al.*, 2005). As this study has shown that lectin ligands have the ability to induce $CD8^+$ T cell responses, against an exogenous antigen, it will be of interest to determine the role of lectin receptors and TLR in the induction of cross-priming.

Accordingly, the aim of the work presented here was in the first instance to determine the relevance of the IFN-α/β licensing pathway in the induction of cross-priming by TLR agonists. The second part of the work was to investigate both the contributions of CD40/CD40L and IFN-α/β licensing pathways in the induction of cross-priming by high-mannose molecules. The role of the mannose receptor and of TLR4 was also examined.

5.2 Mechanisms of induction of cross-priming by Toll-like receptor agonists

Induction of antigen-specific adaptive responses relies on a chain of signals that is initiated by the innate immune system. Early events involve the production of innate cytokines, which may act directly on adaptive cells, and/or indirectly through activation of other innate cells. The production of cytokines in response to TLR agonists has mainly been studied *in vitro*, on bone marrow-derived or purified populations of DC. In order to characterise the response to TLR stimuli in an integrated system, the production of innate cytokines *in vivo* was assessed.

5.2.1 Systemic pro-inflammatory mediators

C57Bl/6 mice were injected i.m. with individual TLR agonists. Serum levels of various pro-inflammatory mediators were measured over a time-course, using flow cytometry array (Figure 5.1). In response to most agonists, major pro-inflammatory cytokines, such as TNF-α and IL-6, were produced in large quantities as rapidly as 2 h after injection. Production of TNF-α and IL-6, however, was not sustained, since by 12 h, serum concentrations had decreased rather dramatically. All agonists

induced high levels of macrophage chemoattractant MCP-1, a chemokine critical against some bacterial and viral infections (Nakano *et al.*, 1994; Hokeness *et al.*, 2005). Production of MCP-1 was long-lasting, especially in the case of poly(I:C), LPS from *E. coli* and CpG 1668, with which chemokine concentration was still above 1,000 pg/mL 12 h after injection. The kinetic of MCP-1 induction by CpG 2216 suggested that this agonist may elicit delayed production of pro-inflammatory mediators compared to the other agonists, a trend that was also observed for IL-6 production. Indeed, IL-6 and MCP-1 concentrations, although much lower than with CpG 1668 for example, increased over the 12 h time-course. Serum levels of the bioactive form of IL-12p70, a key cytokine in the initiation of effective cellular responses, were also measured. Results show that the only high-inducer of IL-12p70 was CpG 1668. A short lasting production of 100-200 pg/mL was detected in response to R-848. IFN-γ is also a relevant effector cytokine. Only R-848 and CpG 2216 seemed to be able to induce appreciable levels of IFN-γ. While concentration peaked suddenly and briefly at 4 h with R-848, it is not known whether CpG 2216 would induce higher and/or lasting production of IFN-γ beyond 12 h after injection.

In summary, all agonists were able to induce often very high serum levels of pro-inflammatory mediators during the first 12 h after injection. However, cytokines involved in activation of cellular immune responses were detected systemically in response to R-848 and CpG DNA only.

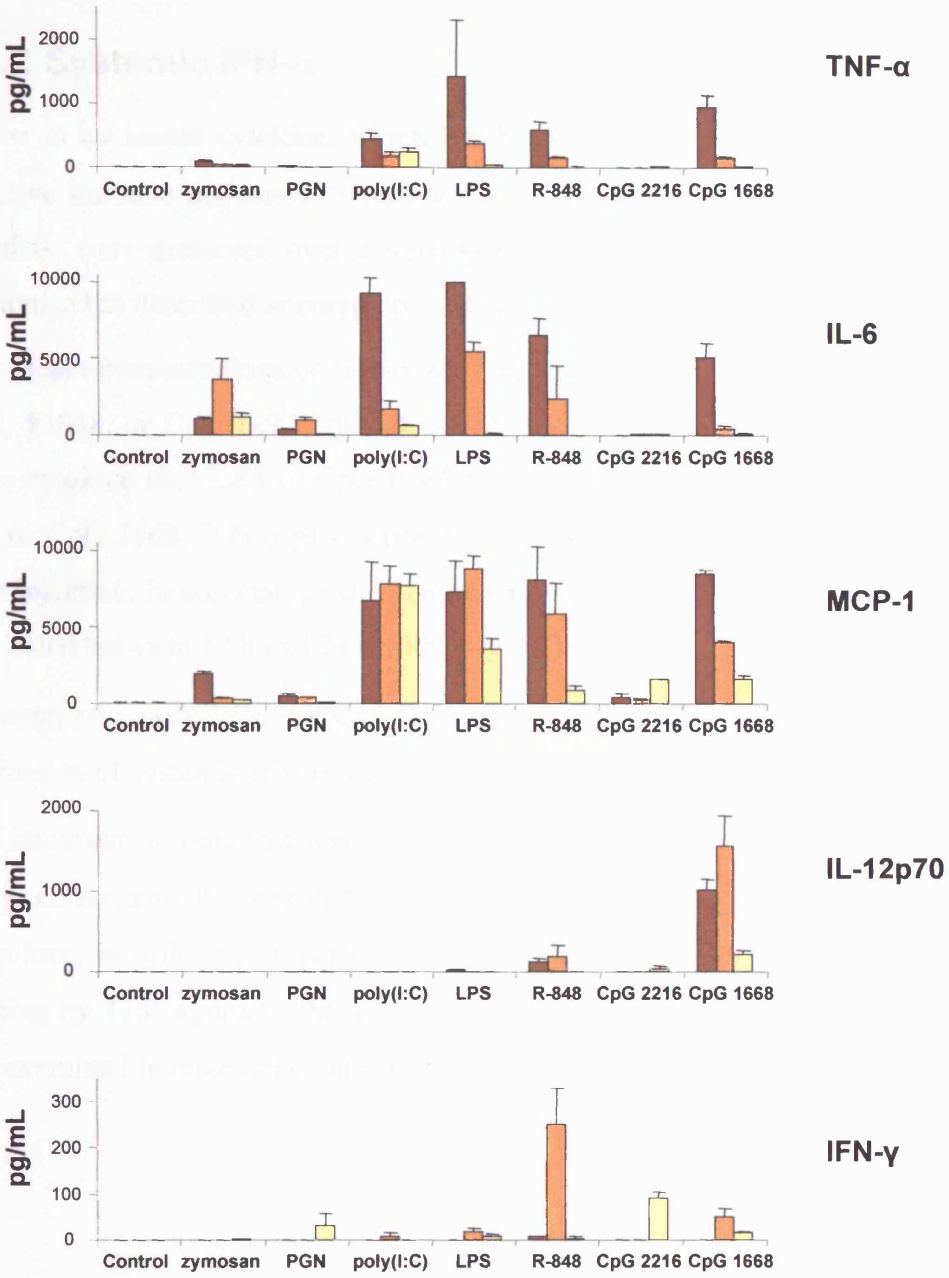

Figure 5.1. TLR agonists rapidly induce high levels of pro-inflammatory mediators in the serum. C57Bl/6 mice were injected i.m. with zymosan (5 mg), peptidoglycan from *S. aureus* (PGN) (200 μg), poly(I:C) (100 μg), LPS from *E. coli* (10 μg), R-848 (200 μg), CpG 2216 (20 nmol) or CpG 1668 (20 nmol). Sera were collected at 2 h (■), 4 h (■) and 12 h (□) after immunisation. TNF-α, IL-6, MCP-1, IL-12p70 and IFN-γ were quantified in the serum of each mouse using flow cytometry array (mouse inflammation cytometric bead array; BD Biosciences). Data are represented by mean ± SD for three mice per group.

5.2.2 Systemic IFN-α

IFN-α is an innate cytokine, which has been shown to stimulate antigen-specific adaptive immune responses. Serum levels of IFN-α, produced in response to TLR agonists, were measured over a time-course, using ELISA (see 2.4.2). Mice were immunised as described in paragraph 5.2.1.

Significant concentrations of IFN-α were detected 2 h after injection with poly(I:C), LPS, R-848, or CpG DNA, though R-848 and poly(I:C) induced at least 10 times more cytokine than LPS or CpG DNA (Figure 5.2). In mice injected with, LPS, R-848 or CpG 1668, IFN-α production returned to baseline concentration by 12-24 h after injection. In contrast, production was constant for at least 24 h with CpG 2216, and lasted between 12 h and 24 h with poly(I:C).

In summary, poly(I:C), LPS, R-848 and CpG DNA were able to induce the production of systemic IFN-α, as opposed to zymosan and peptidoglycan.

It is important to note that levels of IFN-α only were measured. At the time of this study, no reagents that enable the measure of serum levels of IFN-β were available. Therefore, in order to properly assess the role of IFN-α/β in induction of cross-priming by TLR agonists, the generation of antigen-specific $CD8^+$ T cell responses was examined in mice deficient for the IFN–α/β receptor.

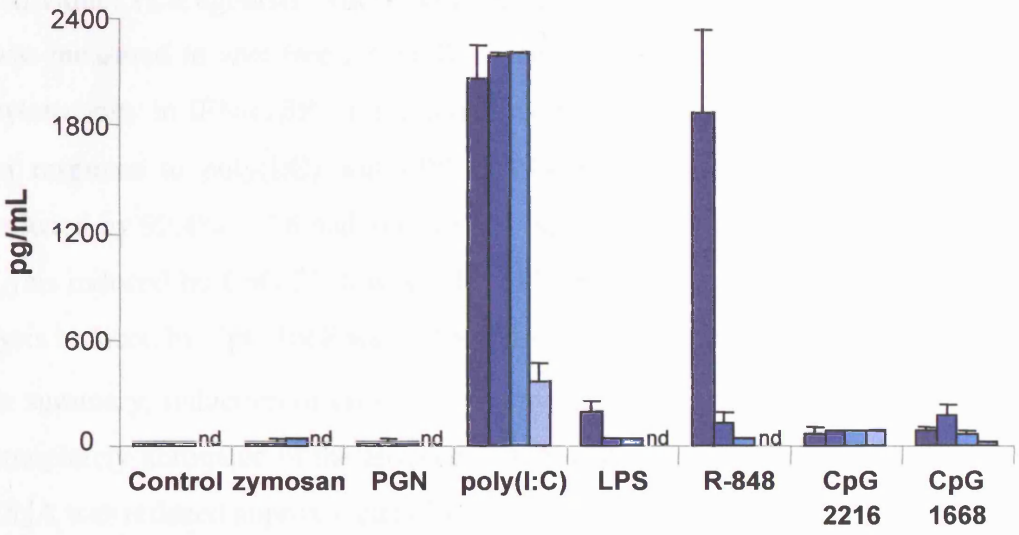

Figure 5.2. Measurement of serum levels of IFN-α induced by TLR agonists. C57Bl/6 mice were injected i.m. with zymosan (5 mg), peptidoglycan from *S. aureus* (PGN) (200 μg), poly(I:C) (100 μg), LPS from *E. coli* (10 μg), R-848 (200 μg), CpG 2216 (20 nmol) or CpG 1668 (20 nmol). Sera were collected at 2 h (■), 4 h (■), 12 h (■) and 24 h (■) after immunisation. IFN-α was quantified in the serum of each mouse using ELISA. Data are represented by mean ± SD for three mice per group. nd, not done.

5.2.3 Antigen-specific $CD8^+$ T cell responses in the absence of IFN-α/β signalling.

5.2.3.1 Responses to Toll-like receptor agonists

In order to characterise more directly the contribution of IFN-α/β signalling pathway in the induction of cross-priming by TLR agonists, generation of antigen-specific $CD8^+$ T cell responses against model protein antigen OVA was studied in mice deficient for the IFN-α/β receptor (IFN-α/β$R^{-/-}$). Wild-type 129Sv/Ev mice (WT) and IFN-α/β$R^{-/-}$ mice were immunised i.m. with OVA in combination with individual TLR agonists. Nine days after injection, $OVA_{SIINFEKL}$-specific cytotoxicity was measured *in vivo* (see 2.6.3). Data are expressed as the percentage reduction in cytotoxicity in IFN-α/β$R^{-/-}$ mice compared to WT mice (see 2.6.3.2) (Figure 5.3A). In response to poly(I:C) and LPS, OVA-specific lysis in IFN-α/β$R^{-/-}$ mice was reduced by 92.4% ± 7.6 and 100% ± 0, respectively, compared to lysis in WT mice. Lysis induced by CpG 2216 was reduced by 66.5% ± 29.1 in IFN-α/β$R^{-/-}$ mice, and lysis induced by CpG 1668 was reduced by 57.6% ± 16.8.

In summary, induction of cross-priming by poly(I:C) and LPS was totally or almost completely abrogated in the absence of IFN-α/β signalling, while the effect of CpG DNA was reduced approximately by half.

5.2.3.2 Responses to non-classical lipopolysaccharides

The role of IFN-α/β in the induction of cross-priming by non-classical lipopolysaccharides was also studied. Wild-type 129Sv/Ev mice (WT) and IFN-α/β$R^{-/-}$ mice were immunised i.m. with OVA in combination with LPS from *E. coli* (Ec O55:B5), LPS from *K. pneumoniae* (Kp K52), LPS from *N. meningitidis* (Nm 44/76). Nine days after injection, SIINFEKL-specific cytotoxicity was measured *in vivo*. Data are expressed as the percentage reduction in cytotoxicity in IFN-α/β$R^{-/-}$ mice compared to WT mice (Figure 5.3B).

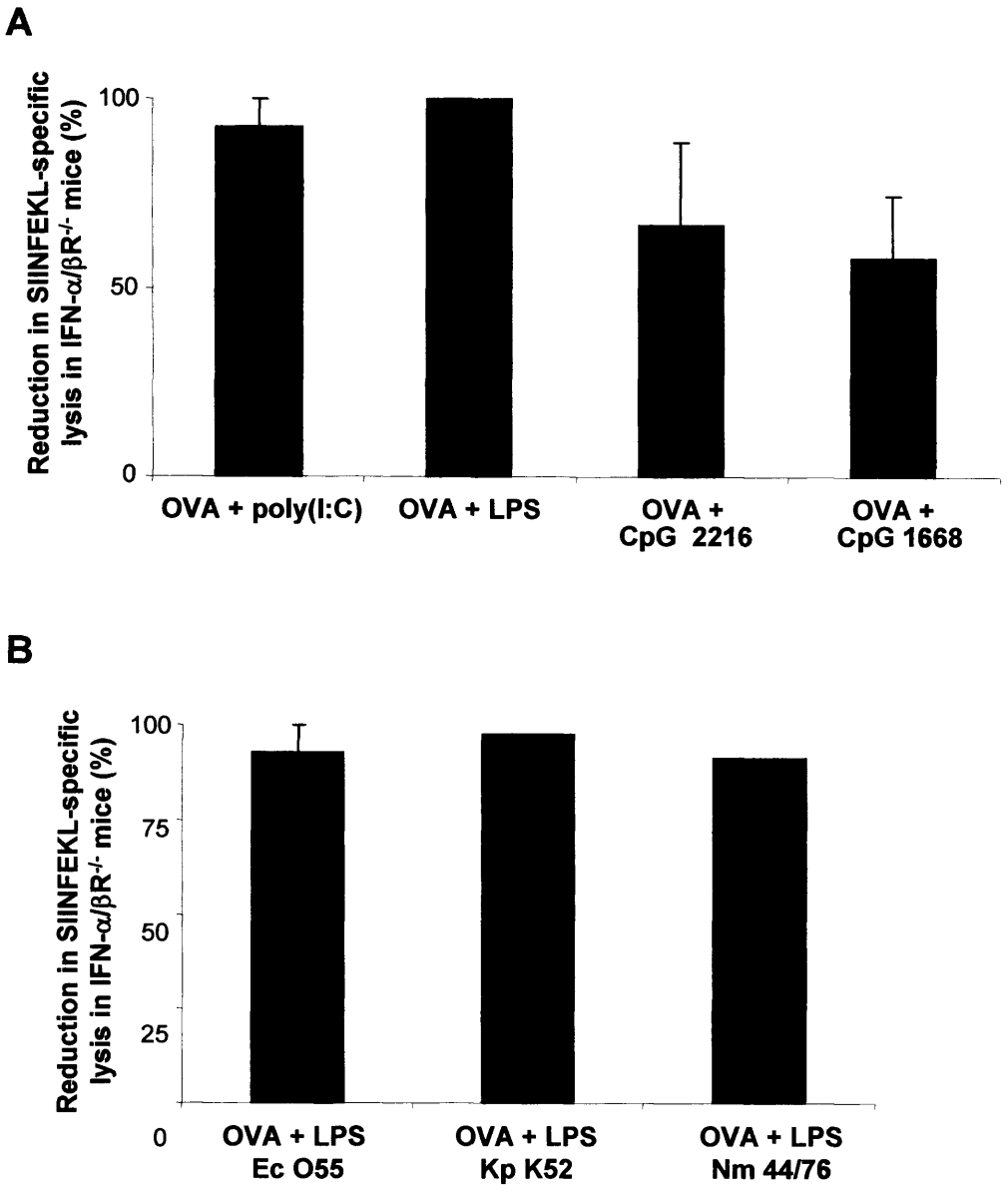

Figure 5.3. Induction of cross-priming by TLR agonists is dependent on the IFN-α/β pathway. (A) Wild-type (WT) 129 Sv/Ev mice and IFN-α/βR-deficient (IFN-α/βR$^{-/-}$) mice were injected i.m. with 500 μg ovalbumin (OVA) in combination with poly(I:C) (100 μg), LPS from *E. coli* (10 μg), CpG 2216 (20 nmol) or CpG 1668 (20 nmol). Nine days after immunisation, OVA-specific CD8$^+$ T cell cytotoxic activity was determined by *in vivo* CTL assay. Results are expressed as percentage of reduction in SIINFEKL-specific lysis in IFN-α/βR$^{-/-}$ mice compared to WT mice. Data are represented by mean ± SD for three mice per group. (B) WT mice and IFN-α/βR$^{-/-}$ mice were injected i.m. with 500 μg OVA alone or OVA in combination with LPS from *E. coli* O55:B5 (Ec 055) (10 μg), LPS from *K. pneumoniae* K52 (Kp K52) (10 μg) or LPS from *N. meningitidis* 44/76 (Nm 44/76) (10 μg). Nine days after immunisation, OVA-specific CD8$^+$ T cell cytotoxic activity was determined by *in vivo* CTL assay. Results are expressed as percentage of reduction in SIINFEKL-specific lysis in IFN-α/βR$^{-/-}$ mice compared to WT mice. Data are represented by mean ± SD for three mice per group.

SIINFEKL-specific lysis induced by each LPS was reduced by over 90% in IFN-α/βR$^{-/-}$ mice compared to lysis in WT mice. Induction of OVA-specific cytotoxicity by LPSs from various bacterial sources was thus entirely dependent on the presence of IFN-α/β signalling.

In summary, poly(I:C), which is a TLR3 agonist, lipopolysaccharides that (most probably) are TLR4 agonists, and CpG DNA, which is a TLR9 agonist, are structures that induce antigen-specific CD8$^+$ T cell responses, a property that was totally abrogated, or partially in the case of CpG DNA, in the absence of IFN-α/β signalling.

5.3 Investigation of mechanisms involved in induction of cross-priming by mannan and high-mannose structures

Experimental data presented in chapter 4 showed that mannan and other high-mannose molecules were able to induce functional cross-priming. With the aim of characterising the possible mechanisms involved, established licensing pathways, such as CD40L/CD40 ligation and IFN-α/β signalling, were investigated. The roles of CD4$^+$ T cells and of IL-12, which is a major T helper cell-inducer, were also determined. Cross-priming, in response to mannan and high-mannose structures, was studied against OVA, using various murine knock-out models.

5.3.1 Licensing pathways

5.3.1.1 The role of CD4$^+$ T cell help, IL-12 and CD40/CD40L interaction

Mannan and high-mannose structures were found to act as adjuvants in the development of antigen-specific CD4$^+$ T cell responses [see 4.3.2], which may constitute an appropriate environment for induction of cross-priming. Furthermore, there has been evidence for cross-priming requiring help from CD4$^+$ T cell (Bennett *et al.*, 1997), although other models of cross-priming proved not to be dependent on CD4$^+$ T cells (Le Bon *et al.*, 2003). Thus, the involvement of CD4$^+$ T cells, and potential associated signalling events, in stimulating antigen-specific CD8$^+$ T cell responses was evaluated in parallel.

Since MHC II molecules are required for $CD4^+$ T cells positive selection in the thymus, MHC II-deficient mice lack $CD4^+$ T cells. Therefore, in order to determine directly the role of $CD4^+$ T cells in induction of cross-priming by high-mannose compounds, experiments were conducted using MHC II-deficient mice (Figure 5.4A). Wild-type C57Bl/6 mice (B6) and MHC II-deficient mice (MHC $II^{-/-}$) were immunised i.m. with OVA in combination with mannan, polymannose LPS from *K. pneumoniae* K55 (LPS Kp K55) or rough LPS from *K. pneumoniae* K52 (LPS Kp K52). Nine days after injection, SIINFEKL-specific cytotoxicity was measured *in vivo*. Mannan, LPS Kp K55 and LPS Kp K52 were still able to induce OVA-specific cytotoxicity in the absence of $CD4^+$ T cells. Lysis of target cells in MHC $II^{-/-}$ mice was actually increased, possibly due to a lack of regulatory T cells (Haeryfar *et al.*, 2005). Indeed, for example, the percentage lysis induced by mannan was 92.1% ± 1.7, compared to 18.9% ± 7.6 in B6 mice.

IL-12 is an early innate cytokine that plays a predominant role in the differentiation of naive $CD4^+$ T cells into Th1 T cells (Macatonia *et al.*, 1995), and which may also promote CTL responses (Sad *et al.*, 1995; Bianchi *et al.*, 1996). Furthermore, IL-12 has been shown to participate in induction of cross-priming against a soluble antigen (Van Uden *et al.*, 2001; Cho *et al.*, 2002). In order to test how essential IL-12 is in the stimulation of cross-priming by mannan and polymannose LPS Kp K55, experiments were conducted in IL-12p35-deficient mice (IL-$12p35^{-/-}$). Wild-type B6 mice and IL-$12p35^{-/-}$ mice were immunised i.m. with OVA in combination with mannan or LPS Kp K55. Nine days later, SIINFEKL-specific cytotoxicity was measured *in vivo* (Figure 5.4B). Percentages of OVA-specific lysis were similar in wild-type and IL-$12p35^{-/-}$ mice, for both mannan and LPS Kp K55. Biologically active IL-12 (IL-12p70) necessitates the combination of two subunits, including IL-12p35; therefore results showed that lack of functional IL-12 did not significantly impair the ability of mannan and LPS Kp K55 to induce OVA-specific $CD8^+$ T cell responses.

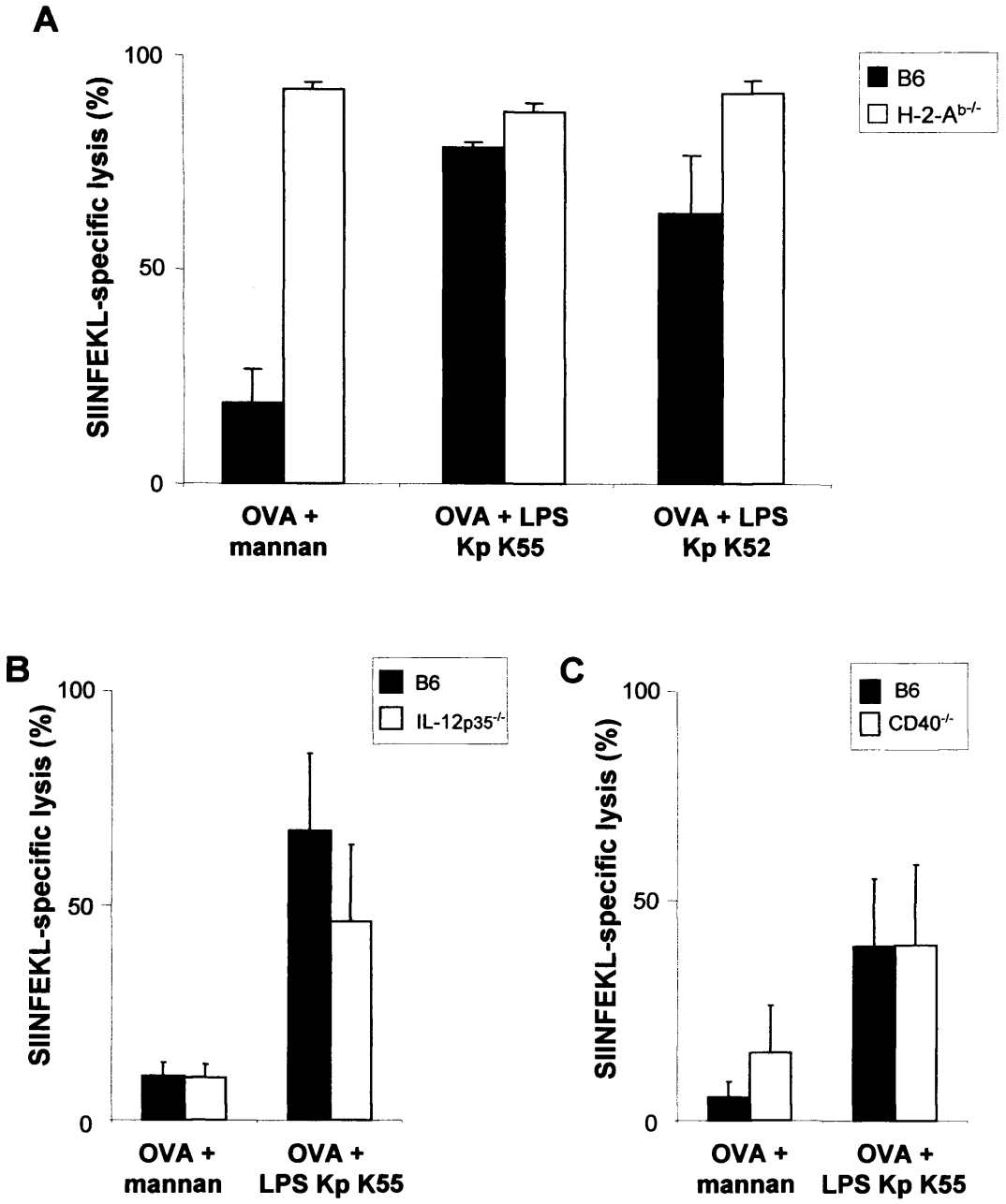

Figure 5.4. Induction of cross-priming by TLR-agonists does not depend on CD4$^+$ T cell help, nor IL-12, nor CD40/CD40L signalling. (A) C57Bl/6 (B6) mice and MHC II-deficient (H-2-A$^{b-/-}$) mice were injected i.m. with 500 μg OVA in combination with mannan from *S. cerevisiae* (2.8 mg), LPS from *K. pneumoniae* O3:K55 (Kp K55) (10 μg) or LPS from *K. pneumoniae* K52 (Kp K52) (10 μg). (B) B6 mice and IL-12p35-deficient (IL-12p35$^{-/-}$) mice were injected as in (A). (C) B6 and CD40-deficient (CD40$^{-/-}$) mice were injected as in (A). (A, B, C) Nine days after immunisation, SIINFEKL-specific CD8$^+$ T cell cytotoxic activity was determined by *in vivo* CTL assay. Results are expressed as percentage of SIINFEKL-specific lysis. Data are represented by mean ± SD for three mice per group.

As described in Chapter 1.2.2.3.2, ligation of CD40L, on T cells, with CD40, on APC, may allow DC, which are able to cross-present exogenous antigens, to become competent for inducing functional cross-priming. It has been previously reported that licensing for cross-priming may be dependent on CD40/CD40L interaction (Bennett *et al.*, 1998; Schoenberger *et al.*, 1998), or not (Cho *et al.*, 2002; Le Bon *et al.*, 2003). Therefore, to test the dependence of high-mannose stimuli on CD40/CD40L ligation as a licensing factor, the ability of mannan and LPS Kp K55 to generate cross-priming against OVA was tested in mice lacking CD40 molecules (Figure 5.4C). Wild-type B6 mice and CD40-deficient mice ($CD40^{-/-}$) were immunised i.m. with OVA in combination with mannan or LPS Kp K55. Nine days later, SIINFEKL-specific cytotoxicity was measured *in vivo*. Results showed that mannan and LPS Kp K55 were still able to induce OVA-specific cytolysis in the absence of CD40.

In summary, mannan and both LPS Kp K55 and Kp K52 induced cross-priming despite the absence of $CD4^+$ T cells. In addition, mannan and LPS Kp K55 did not depend on CD40 or IL-12 to generate antigen-specific $CD8^+$ T cell responses.

5.3.1.2 IFN-α/β signalling

IFN-α/β signalling is a major licensing pathway for the initiation of cross-priming. Since induction of cross-priming by mannan and polymannose LPS Kp K55 did not depend on other prominent licensing pathways, as shown in paragraph 5.3.1.1, the role of the IFN-α/β signalling pathway required to be investigated. In order to test whether high-mannose structures were able to induce cross-priming in the absence of IFN-α/β signalling, experiments were conducted in IFN-α/β$R^{-/-}$mice (Figure 5.5A).

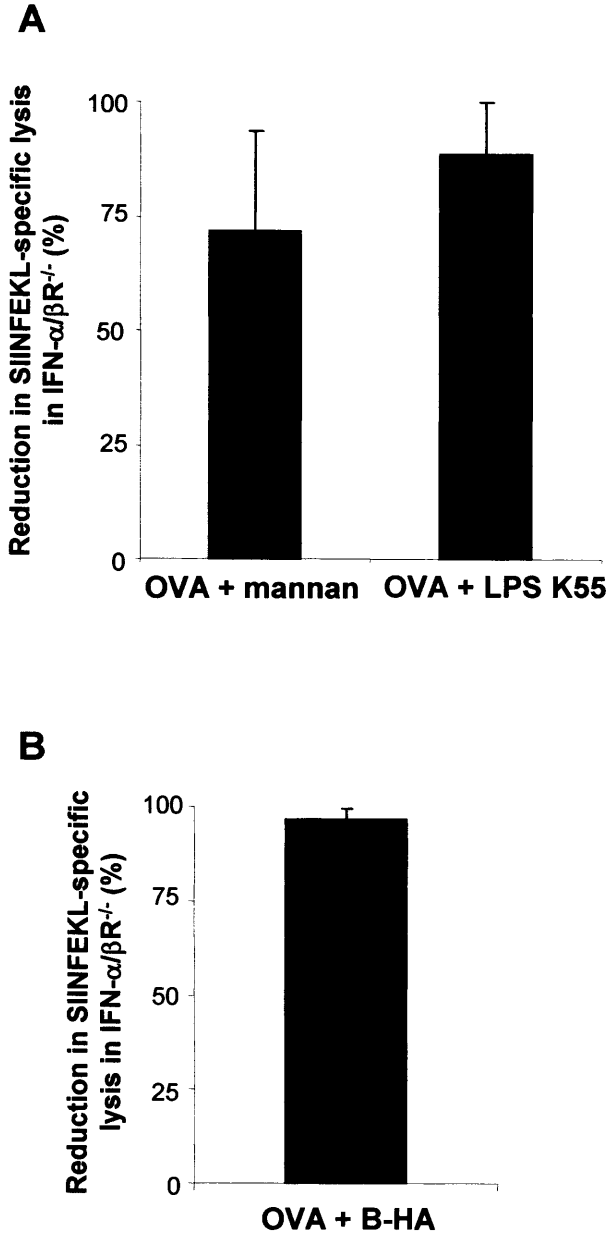

Figure 5.5. The role of IFN-α/β in induction of cross-priming by mannan and high-mannose compounds. (A, B) Wild-type (WT) 129 Sv/Ev mice and IFN-α/βR-deficient (IFN-α/βR$^{-/-}$) mice were injected i.m. with 500 μg OVA in combination with mannan from *S. cerevisiae* (2.8 mg) or LPS from *K. pneumoniae* O3:K55 (Kp K55) (10 μg). (A) Nine days after immunisation, SIINFEKL-specific CD8^{+} T cell cytotoxic activity was determined by *in vivo* CTL assay. Results are expressed as percentage of reduction in SIINFEKL-specific lysis in IFN-α/βR$^{-/-}$ mice compared to WT mice. Data are represented by mean ± SD for three mice per group. (B) WT mice and IFN-α/βR$^{-/-}$ mice were injected i.m. with OVA (500 μg) in combination with bromelain-released hemagglutinin (B-HA) from Influenza X:31 A/AICHI/68 (20μg). Nine days after immunisation, SIINFEKL-specific CD8^{+} T cell cytotoxic activity was determined by *in vivo* CTL assay. Results are expressed as percentage of reduction in SIINFEKL-specific lysis in IFN-α/βR$^{-/-}$ mice compared to WT mice. Data are represented by mean ± SD for three mice per group.

Wild-type 129Sv/Ev mice (WT) and IFN-α/βR$^{-/-}$ mice were immunised i.m. with OVA in combination with mannan or LPS Kp K55, and OVA-specific CD8$^+$ T cell cytotoxicity was assessed nine days later *in vivo*. Data are expressed as percentage reduction in SIINFEKL-specific lysis in IFN-α/βR$^{-/-}$ mice compared to wild-type mice. Results showed that SIINFEKL-specific lysis in response to mannan was reduced by 71.8% ± 22.0 in IFN-α/βR$^{-/-}$ mice compared to lysis in WT mice. The cytotoxic response induced by LPS Kp K55 was also greatly reduced in IFN-α/βR$^{-/-}$ mice (by 88.7%± 11.0).

Hemagglutinin (HA) from Influenza A virus (H3N2) was found to have the adjuvant property of inducing cross-priming against a soluble protein antigen (see 4.3.1.2.2). The role of IFN-α/β signalling in this process was examined (Figure 5.5B). Wild-type 129Sv/Ev mice (WT) and IFN-α/βR$^{-/-}$ were immunised i.m. with OVA in combination with HA, and OVA-specific cytotoxicity was determined nine days later *in vivo*. SIINFEKL-specific lysis induced by HA was significantly reduced in IFN-α/βR$^{-/-}$ mice (by 96.5% ± 3.4) compared to lysis in WT mice.

In summary, initiation of antigen-specific CD8$^+$ T cell responses by high-mannose molecules such as mannan, HA and LPS Kp K55 depended heavily on IFN-α/β signalling pathway.

In addition to characterising the contribution of established cross-priming licensing pathways, the role of relevant innate receptors, which would be first to recognise the high-mannose molecules studied here, was examined.

5.3.2 Role of innate receptors

5.3.2.1 The mannose receptor

The mannose receptor (MR) is the principal mannose-binding receptor (Stahl *et al.*, 1998; Weis *et al.*, 1998; Geijtenbeek *et al.*, 2004). Ligand-receptor interactions have been implicated in the induction of expression and secretion of immunomodulatory cytokines, including IFN-α (Garner *et al.*, 1994; Yamamoto *et al.*, 1997; Milone *et al.*, 1998). Thus, considering the role of MR in key steps of immune responses to high-mannose molecules (Sallusto *et al.*, 1995; Prigozy *et al.*, 1997; Martinez-Pomares *et al.*, 1998), the MR contribution to the induction of cross-priming by

mannan and polymannose LPS Kp K55 was assessed. Moreover, with the aim to refine the characterisation of polymannose LPS properties, polymannose LPS from *E. coli* O9 was included in the study. Indeed, both LPS Ec O9 and LPS Kp K55 have mannose homopolymer O-antigen polysaccharide chains (Curvall *et al.*, 1973; Prehm *et al.*, 1976; Chen *et al.*, 2002). In contrast, LPS Kp K52 and LPS Ec O55 do not contain a polymannose O-antigen. Wild-type B6 mice (WT) and MR-deficient mice ($MR^{-/-}$) were immunised i.m. with OVA alone or OVA in combination with mannan, LPS Kp K55, LPS Kp K52, LPS Ec O9 or LPS Ec O55. To first assess whether the absence of MR affected the ability of the compounds to induce proliferation of OVA-specific $CD8^+$ T cells, the frequency of $CD8^+$ T cell bearing SIINFEKL-specific TCR was measured in peripheral blood eight days after immunisation, using H-$2K^b$-SIINFEKL tetramer staining and FACS analysis (Figure 5.6A). Results showed that LPS Kp K52, LPS Ec O55 stimulated the expansion of OVA-specific $CD8^+$ T cells in both WT and $MR^{-/-}$ mice. Mannan and LPS Ec O9 properties were not affected either in $MR^{-/-}$ mice compared to WT mice. However, less OVA-specific $CD8^+$ T cells were detected in $MR^{-/-}$ mice immunised with OVA + LPS Kp K55, in comparison to WT mice (0.86% ± 0.16 in WT compared to 0.39% ± 0.08 in $MR^{-/-}$; p=0.006). In order to test the functional properties of OVA-specific $CD8^+$ T cells generated in $MR^{-/-}$ mice, in comparison to WT mice, CTL assays *in vivo* were conducted (Figure 5.6B). Percentages of SIINFEKL-specific lysis induced by LPS Kp K52 and LPS Ec O55 in $MR^{-/-}$ mice were not significantly different from percentages of lysis measured in WT mice. Similarly, OVA-specific-lysis induced by mannan, LPS Kp K55 and LPS Ec O9 were statistically comparable in WT and in $MR^{-/-}$ mice.

Overall, results showed that lack of MR did not interfere significantly with the induction of functional antigen-specific $CD8^+$ T cell responses by high-mannose structures.

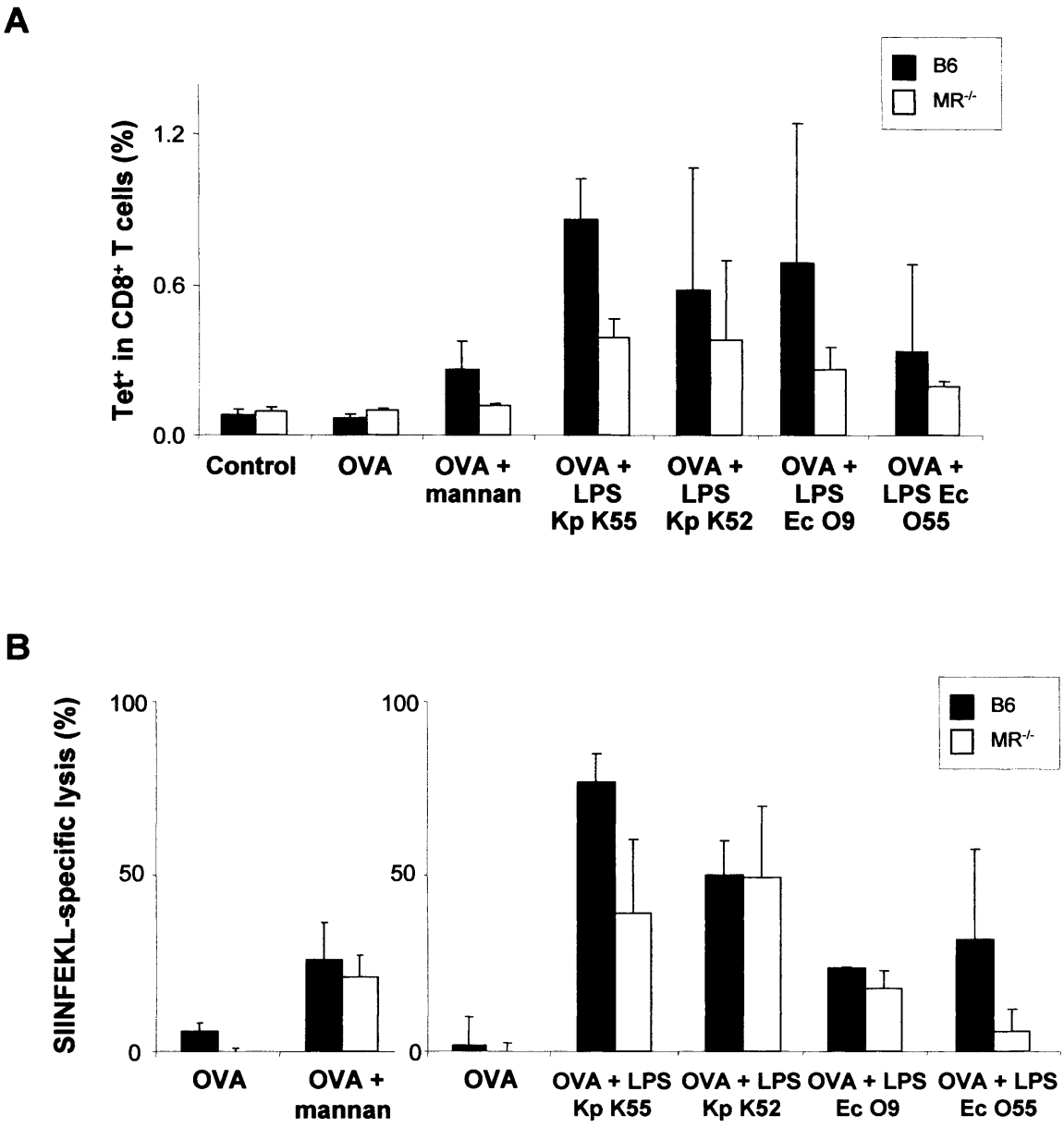

Figure 5.6. The mannose receptor is not required for induction of cross-priming by mannan and polymannose lipopolysaccharides. C57Bl/6 mice and MR-deficient ($MR^{-/-}$) mice were injected i.m. with 500 μg OVA alone or OVA in combination with mannan from *S. cerevisiae* (2.8 mg), LPS from *K. pneumoniae* O3:K55 (Kp K55) (10 μg), LPS from *K. pneumoniae* K52 (Kp K52) (10 μg), LPS from *E. coli* O9:K9(L9):H12 (Ec O9) (10 μg) or LPS from *E. coli* O55:B5 (Ec 055) (10 μg). (A) Eight days after immunisation, SIINFEKL-specific $CD8^+$ T cells in peripheral blood were quantified using K^b-SIINFEKL tetramer staining. Results are expressed as percentage of Tet^+ cells gated on $CD8^+$ T cells. Data are represented by mean ± SD for three mice per group. (B) Nine days after immunisation, SIINFEKL-specific $CD8^+$ T cell cytotoxic activity was determined by *in vivo* CTL assay. Results are expressed as percentage of SIINFEKL-specific lysis. Data are represented by mean ± SD for three mice per group.

5.3.2.2 Toll-like receptors

Toll-like receptors (TLR) are a group of receptors that recognise specific structures from, among others, bacteria, fungi, and viruses. Stimulation of TLR signalling pathways results in the transcription of precise sets of genes encoding for cytokines and costimulatory molecules. TLR4 in particular has been shown to initiate signalling cascades that can lead to the production of immunomodulatory cytokines such as IFN-β and/or influence the expression of CD40 on APC (Toshchakov *et al.*, 2002; Hoebe *et al.*, 2003b; Nicolle *et al.*, 2004). Since both mannan and LPS from *E. coli* have been described as TLR4 agonists (Hoshino *et al.*, 1999; Tada *et al.*, 2002), and TLR4 may participate in the initiation of cross-priming licensing pathways, the contribution of TLR4 in induction of antigen-specific $CD8^+$ T cell responses by mannan and polymannose LPS Kp K55 was assessed. Wild-type C57Bl/10 mice (WT) and TLR4-deficient mice ($TLR4^{-/-}$) were immunised i.m. with model protein antigen OVA alone, or OVA in combination with mannan, LPS Kp K55 or LPS Ec O55. Nine days after injection, OVA-specific cytotoxicity was measured *in vivo*. Data from the CTL assay *in vivo* are expressed as the percentage reduction in SIINFEKL-specific lysis in $TLR4^{-/-}$ mice compared to WT mice (Figure 5.7). Results showed that OVA-specific lysis in $TLR4^{-/-}$ mice, in response to mannan or LPS Ec O55, was reduced by 76.0% ± 13.8 and 89.0% ± 10.1, respectively, compared to cytolysis in WT mice. In sharp contrast, OVA-specific lysis generated by LPS Kp K55 was at similar levels in both WT and $TLR4^{-/-}$ mice.

In summary, induction of antigen-specific $CD8^+$ T cell responses by mannan or LPS Ec O55 was TLR4-dependent, whereas induction by LPS Kp K55 was TLR4-independent.

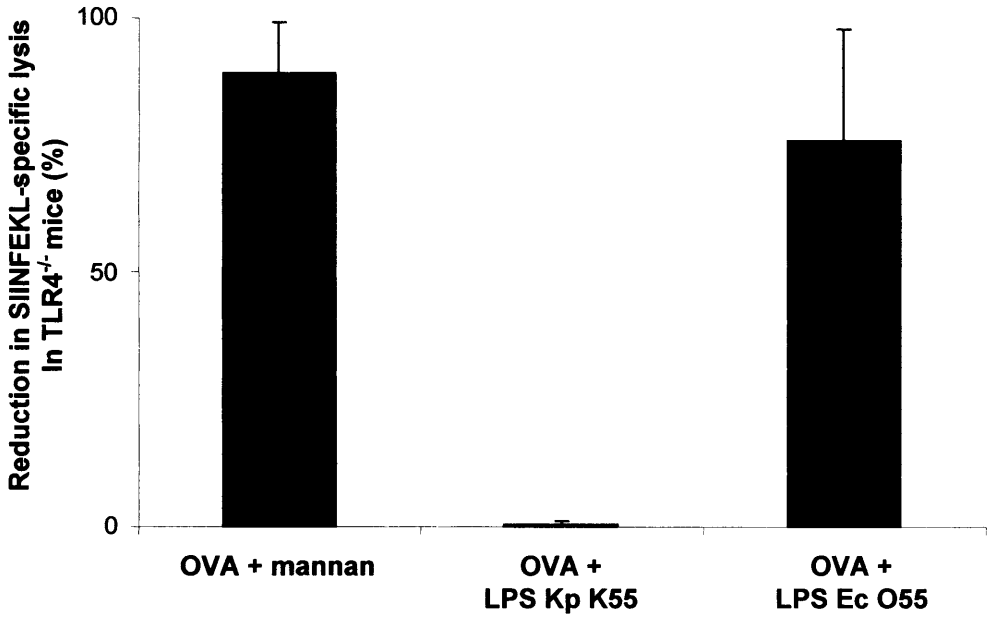

Figure 5.7. The role of TLR4 in induction of cross-priming by mannan and lipopolysaccharides. Wild-type (WT) C57Bl/10ScSnJ mice and TLR4-deficient ($TLR4^{-/-}$) mice were injected i.m. with 500 μg OVA in combination with mannan from *S. cerevisiae* (2.8 mg), LPS from *K. pneumoniae* O3:K55 (Kp K55) (10 μg) or LPS from *E. coli* O55:B5 (Ec 055) (10 μg). Nine days after immunisation, OVA-specific $CD8^+$ T cell cytotoxic activity was determined by *in vivo* CTL assay. Results are expressed as percentage of reduction in SIINFEKL-specific lysis in $TLR4^{-/-}$ mice compared to WT mice. Data are represented by mean ± SD for three mice per group.

5.4 Conclusions and discussion

Activation of $CD8^+$ T cell cytotoxic programs needs to be controlled to prevent autoimmunity. DC licensing constitutes a safety protocol in the initiation of cross-priming, in the sense that $CD8^+$ T cells can only become functional if they are actively instructed to do so by competent DC. The precise nature and effects of DC signals acting on $CD8^+$ T cells have not been fully elucidated, and what a DC competent state entails has also not been entirely defined. However, CD40/CD40L interactions and IFN-α/β signalling have been shown to be two major upstream licensing stimuli, which ultimately allow induction of cross-priming. In chapters 3 and 4, particular PAMPs were proven to induce cross-priming against a soluble protein antigen. The aim of the work presented in this chapter was to investigate the nature of the licensing stimuli involved. Results indicate that for PAMPs induction of cross-priming against a soluble antigen, the IFN-α/β licensing pathway is dominant.

Of a range of representative TLR agonists, only poly(I:C) (TLR3), LPS from *E. coli* (TLR4) and CpG DNA (TLR9) were found able to induce cross-priming against a soluble protein antigen (see Chapter 3). The hypothesis tested in the present chapter was that IFN-α/β played an essential part in the licensing process.

TLR agonists, as PAMPs in general, are known to be detected rapidly, and part of the response they elicit is the production of cytokines, which act as alarm signals and activation mediators. Indeed, all TLR agonists tested *in vivo* stimulated systemic release of high levels of cytokines as early as 2 h after intramuscular injection. However, while poly(I:C) could induce the production of over 2 ng/mL IFN-α, LPS and CpG DNA generated a substantial (up to 250 pg/mL) yet much lower response. On the other hand, R-848, a TLR7 agonist that was shown to be unable to generate antigen-specific $CD8^+$ T cell responses against a soluble protein antigen (see Chapter 3), nevertheless briefly induced very high serum levels of IFN-α (2 ng/mL) 2 h after injection. If secretion of IFN-α did not seem to define a specific connection between IFN-α/β and the ability of poly(I:C), LPS and CpG DNA to induce cross-priming, experiments with knock-out mice lacking the IFN-α/β receptor proved that induction of cross-priming by those TLR agonists was dependent on IFN-α/β signalling, albeit to a varying degree. Induction of cross-priming by poly(I:C) and LPS from *E. coli* was completely dependent on IFN-α/β, while induction of cross-priming by CpG

DNA was only partially dependent, which is consistent with a previous report showing that induction of cross-priming by CpG DNA is dependent on IFN-α/β, IL-12 and costimulatory molecules (Van Uden *et al.*, 2001; Cho *et al.*, 2002). Therefore, measuring IFN-α/β production may provide information on the types of innate signalling pathways activated by agonists, but cytokine secretion in early stages of a response may not be predictive of consequent adaptive responses. In addition, in the present study, only levels of IFN-α were measured, using a monoclonal anti-mouse IFN-α capture antibody; binding of this antibody to each IFN-α existing subtype has not been characterised by the manufacturer. The actual specificity of capture antibodies for IFN-α subtypes, as well as their affinity, could result in equally produced IFN-α subtypes not being equally detected; the IFN-α ELISA thus may not reflect accurately the contribution of each IFN-α subtype, which may be differentially induced by different agonists. If particular subtypes were responsible for licensing of cross-priming, then this ELISA does not allow one to establish a correlation between induction of IFN-α production by agonists and induction of cross-priming. This could provide an explanation for the observation that R-848 is a potent inducer of IFN-α, but was not able to induce cross-priming against a soluble protein antigen. It is also to note that the secretion of IFN-α in response to R-848 was short-lasting. Furthermore, stimulation of IFN-β production by agonists, which was not examined because reagents were not commercially available at the time, may play an important role in the cross-priming licensing process. Although poly(I:C), LPS from *E. coli*, R-848 and CpG DNA have all been found to induce the production of IFN-β (Megyeri *et al.*, 1995; Hoshino *et al.*, 2002; Oshiumi *et al.*, 2003a), it is possible that agonists induce different combinations of various IFN-α subtypes and IFN-β that are not equally effective at inducing cross-priming. Overall, it is thus possible that various factors, such as the source, nature, kinetics and magnitude of IFN-α/β response, all concur to license for cross-priming.

While the studies presented here focussed on investigating the mechanisms involved in induction of cross-priming, it is interesting to note that *in vivo* secretion, or lack of, of other innate cytokines in response to TLR agonists did not necessarily correlate either with expected adaptive responses. IL-12p70 for instance is an immunomodulatory cytokine that has been connected, through induction of IFN-γ,

with the promotion of IgG2a isotype switching (Germann *et al.*, 1995; Van Uden *et al.*, 2001). Antigen-specific IgG2a isotype switching can also be dependent on IFN-α/β signalling (Le Bon *et al.*, 2001; Van Uden *et al.*, 2001). While zymosan, poly(I:C), LPS from *E. coli*, R-848, CpG 2216 and CpG 1668 were all found to enhance antigen-specific antibody responses and in particular promote IgG2a production (see Chapter 3), zymosan did not induce the production of IFN-α, and only R-848 and CpG 1668 stimulated the secretion of IL-12 p70. Accordingly, R-848 and CpG 1668 induced the secretion of IFN-γ, and both agonists were reported to induce antigen-specific IgG2a in an IFN-γ-dependent manner, with IgG2a responses being reduced by more than 90% in IFN-γ-deficient mice (Vasilakos *et al.*, 2000). Although CpG 2216 did not stimulate the *in vivo* secretion of IL-12p70, it was the only agonist, other than R-848 and CpG 1168, that was able to induce the production of some IFN-γ, possibly through induction of another immunostimmulatory cytokine such as IL-18 (Bohle *et al.*, 1999; Gould *et al.*, 2004). CpG 2216 and CpG 1668 are two different CpG sequences that were included in the study as representative of two distinct CpG classes: A-class CpG sequences (CpG 2216) have been reported to stimulate the production of high levels IFN-α (Yamamoto *et al.*, 1992; Hemmi *et al.*, 2003; Kerkmann *et al.*, 2003), from plasmacytoid DC in particular (Kadowaki *et al.*, 2001; Krug *et al.*, 2001), and to stimulate NK cells functions such as IFN-γ production and cytotoxicity (Ballas *et al.*, 1996; Vollmer *et al.*, 2004). B-class CpG sequences (CpG 1668) have been reported to primarily stimulate B cell proliferation (Krieg *et al.*, 1995) and the production of cytokines such as IL-6 and IL-10 but only little IFN-α (Yi *et al.*, 1996; Anitescu *et al.*, 1997; Hartmann *et al.*, 1999; Krug *et al.*, 2001). B-type CpG DNA are also reported to induce the production of high-levels of IL-12 (Krieg *et al.*, 1998; Schulz *et al.*, 2000; Vasilakos *et al.*, 2000; Krug *et al.*, 2001). In the present study, comparing the effect of CpG 2216 and CpG 1668 on cytokine secretion *in vivo* confirmed that CpG 1668, as opposed to CpG 2216, was a high inducer of IL-12p70. However, despite being described as a poor inducer of IFN-α, CpG 1668 was also found to be able to stimulate the release of more than 100 pg/mL systemic IFN-α at the peak of the response (4 h after injection), though secretion was not as sustained as the response stimulated by CpG 2216 (over 90 pg/mL IFN-α 24 h after injection).

Cytokines form a network of signal mediators that is complex and redundant. Therefore, studying cytokine production and secretion pattern, whilst providing clues to activation mechanisms at various stages of the immune response, does not necessarily predict the triggering of particular responses such as antigen-specific isotype switching or cross-priming. When possible, it is important to use *in vivo* cytokine or cytokine receptor gene-deficient models to define more accurately the contribution of particular cytokines.

The ability of LPSs from various bacteria species to induce cross-priming against a soluble protein antigen was investigated in Chapter 3. In addition to LPS from *E. coli*, which is widely used as a prototypic LPS, less conventional LPSs such as LPSs from *K. pneumoniae* and *N. meningitidis* were found to induce antigen-specific $CD8^+$ T cell responses. In contrast, LPS purified from a non-enterobacterial species, *P. gingivalis,* which is an LPS with a different lipid A structure, was not able to induce cross-priming. The results reported in the present chapter showed that the enterobacterial LPSs tested, irrelevant of their O-antigen structure, induced cross-priming exclusively in an IFN-α/β-dependent manner.

In addition to studying induction of cross-priming by TLR agonists, including non-classical LPSs, this project involved assessing the ability of another category of PAMPs, that are high-mannose molecules, to generate $CD8^+$ T cell responses against a soluble protein antigen, and examining possible cross-priming licensing mechanisms. Mannan, a component of yeast cell-wall, LPS from *K. pneumoniae* O3:K55, which has a distinct polymannose O-polysaccharide, and hemagglutinin from *Influenza* A virus X:31 (H3N2), an envelope glycoprotein, were all found to be able to induce cross-priming (see Chapter 4). The contribution of various factors, such as $CD4^+$ T cell help, CD40/CD40L ligation and cytokines, in the licensing of cross-priming had been investigated previously for other models. Cross-priming against cell-associated antigen, for instance, requires $CD4^+$ T cell help or CD40/CD40L interaction (Bennett *et al.*, 1997; Bennett *et al.*, 1998), while cross-priming against a soluble antigen in the presence of bacterial products can be mediated by CD4-independent (Bennett *et al.*, 1997; Hamilton *et al.*, 2001), and CD40-independent mechanisms (Cho *et al.*, 2000). IL-12 is a Th1 cytokine that, although not necessarily essential in the licensing of CTL priming (Wan *et al.*, 2001), was shown to be involved in the induction of cross-priming by CpG DNA (Cho *et*

al., 2002). IFN-α, in turn, induces cross-priming against soluble antigen independently of $CD4^+$ T cell help or CD40/CD40L ligation, and overcomes the need for $CD4^+$ T cell help in cross-priming against cell-associated antigen (Le Bon *et al.*, 2003). The work presented in this chapter showed that mannan and polymannose LPS Kp K55 induced cross-priming against a soluble antigen independently of CD40, as well as independently of $CD4^+$ T cell help, which is consistent with studies reporting that $CD4^+$ T cell help in cross-priming is actually mediated by CD40 signalling. Induction of cross-priming by mannan and LPS Kp K55 did not require IL-12 either. In contrast, induction of cross-priming by mannan, LPS Kp K55 and HA was dependent on IFN-α/β signalling. Mannan did not appear to absolutely depend on IFN-α/β signalling for licensing of cross-priming, although it has been reported that other factors, such as CD80-86/CD28 interaction, may be involved in the development of cross-priming (Cho *et al.*, 2002).

LPS, CpG DNA, HA, poly(I:C) and mannan are PAMPs from bacteria, viruses and yeasts. Their detection by the immune system is mediated by different innate receptors. LPS, CpG DNA and poly(I:C) are representative TLR agonists, but can also be recognised by other molecules. LPS-binding protein (LBP), CD14 and TLR4 are all involved in the recognition of LPS from *E. coli*, for example. Bacterial DNA is recognised by TLR9 (Hemmi *et al.*, 2000), but can also bind members of the collectin family, surfactant protein D (SP-D) in particular (Palaniyar *et al.*, 2004), which have their own receptor(s) (Hickling *et al.*, 2004). Poly(I:C) can be recognised by TLR3 (Alexopoulou *et al.*, 2001), but also by PKR (Yang *et al.*, 1995; Balachandran *et al.*, 2000; Diebold *et al.*, 2003) and by the RNA helicases of RIG-1 and mda-5 (Andrejeva *et al.*, 2004; Kato *et al.*, 2005). HA (Reading *et al.*, 1997; Reading *et al.*, 2000) and mannan can be recognised by lectins that have mannose-specific carbohydrate recognition domains, which, for mannan, include mannan-binding lectin (MBL), the mannose receptor and SIGNR1 (Taylor *et al.*, 1992; Takahara *et al.*, 2004; McGreal *et al.*, 2005). Mannan has been shown to interact with TLR4 (Tada *et al.*, 2002).

It has been demonstrated that induction of cross-priming by intracellular poly(I:C) against cell-associated antigen is dependent on TLR3 (Schulz *et al.*, 2005). However, this does not necessarily apply to induction of cross-priming against a soluble antigen. Therefore, the involvement of TLR3 and other receptors for dsRNA would

need to be studied, although extracellular poly(I:C) is probably recognized by TLR3 rather than by other receptors. CpG DNA, either co-administered with or conjugated to a soluble antigen, induces cross-priming (see Chapter 3 and (Cho *et al.*, 2000; Sparwasser *et al.*, 2000). The contribution of TLR9 was investigated, but only in the case of CpG DNA-antigen conjugates and not for co-administered CpG DNA and antigen, and results showed that induction of cross-priming was mediated by TLR9 (Heit *et al.*, 2003).

The mannose receptor (MR) is a C-type lectin with specificity, among others, for D-mannose. It has eight carbohydrate recognition domains (CRD) and clustering of CRD 4 to 8 is thought to allow high-avidity binding to branched-chain mannose oligosaccharides (Taylor *et al.*, 1993; Feinberg *et al.*, 2000). It has been implicated in the recognition of microbial structures, such as yeast mannan and viral glycoproteins (Kery *et al.*, 1992; Miller *et al.*, 2003; Nguyen *et al.*, 2003), and has been shown to bind some LPS structures, including LPS from *K. pneunomiae* O3:K55 (Zamze *et al.*, 2002). In order to assess the contribution of MR in induction of cross-priming by high-mannose molecules, the generation of $CD8^+$ T cell responses against a soluble antigen was examined in MR-deficient mice. Results showed that, generally, induction of cross-priming by mannan and polymannose LPSs from *K. pneumoniae* K55 and from *E. coli* O9 occurred independently of MR. Therefore the MR is not indispensable, in the detection of high mannose molecules and subsequent licensing of cross-priming. The role of the MR in immune responses has been controversial, especially since MR-deficiency did not increase susceptibility of mice to disseminated yeast and fungal infection (Lee *et al.*, 2003c; Swain *et al.*, 2003), although this may only demonstrate that redundant mechanisms exist and/or that responses to β-glucan, rather than mannan, and mediated by a different lectin, such as dectin-1, are more crucial in controlling yeast and fungal infections.

Recognition of high-mannose molecules may be mediated by other C-type lectins with mannose-specificity. SIGNR1 and SIGNR3, for instance, belong to a family of five mouse lectins homologous to human DC-SIGN (Park *et al.*, 2001). Mannan can be recognised by both SIGNR1 and SIGNR3 (Takahara *et al.*, 2004). If SIGNR1 and SIGNR3 may contribute to capturing mannan and high-mannose molecules, induction of cross-priming still requires the initiation of signalling cascades that will result in licensing stimuli being released. Yet the cytoplasmic domain of mouse DC-

SIGN and SIGNR lectins do not contain signalling motifs. Cooperation between receptors has however been demonstrated. For example, LPS-binding protein (LBP) binds LPS for transfer to CD14 and TLR4-MD-2 complex, which transduces appropriate signals (Gioannini *et al.*, 2004; Saitoh *et al.*, 2004; Jiang *et al.*, 2005). Association of SIGNR1 with TLR4-MD-2 complex has also been reported (Nagaoka *et al.*, 2005), which might be involved in the induction of cross-priming by mannan and HA. Indeed, mannan was found to induce TLR4-dependent, polymyxin B-resistant, signalling, (Tada *et al.*, 2002), and results presented in this chapter showed that mannan induced cross-priming against a soluble protein antigen in a TLR4-dependent manner. Results also demonstrated that induction of cross-priming by LPS from *E. coli* was TLR4-dependent. Interestingly though, LPS from *K. pneumoniae* K55 induced cross-priming independently of TLR4. Other LPS molecules have been shown to induce immune responses independently of TLR4. LPS from *P. gingivalis* and LPS from *L. interrogans* for example induce pro-inflammatory cytokine production independently of TLR4 (Kirikae *et al.*, 1999; Hirschfeld *et al.*, 2001; Werts *et al.*, 2001). The adjuvant activity of polymannose LPS from *K. pneumoniae* O3 of inducing antigen-specific DTH and antibody responses was also shown to be TLR4-independent (Ohta *et al.*, 1985).

All data collected indicate that the immune system has the capacity, even with a set number of innate receptors, to detect PAMPs in many various ways, probably by using combinative binding. Yet, diverse recognition mechanisms, dealing with structures originating from different families of organisms, can result in the induction of a same particular downstream adaptive immune response. Hence, PAMPs such as viral DNA, viral and yeast high-mannose carbohydrates, bacterial LPS and bacterial DNA, whether it is through the same innate receptor (LPS from *E. coli* and probably LPS from *N. meningitidis*) or not (LPS from *K. pneumoniae* K55), through TLRs (LPS from *E. coli*, CpG DNA) or yet unknown combination of other receptors, are able to trigger signalling programs that somewhere converge in the induction of IFN-α/β-dependent cross-priming.

Chapter 6
Final Discussion

Microbial infections with intracellular organisms are a cause of morbidity and mortality world-wide, and vaccination is a strategy that would help preventing infection, and therefore its consequences. Vaccines in those cases need to elicit $CD8^+$ T cells that kill infected cells and produce cytokines that support additional killing mechanisms by other cells. Because of concerns over the safety of using live organisms as vaccines, the use of recombinant target antigens is being evaluated instead. $CD8^+$ T cell responses may be elicited against exogenously administered antigens, by cross-priming. Cross-priming is not constitutive, and studying the signals that induce and control cross-priming contributes to the understanding of a process that can be exploited for new safe vaccine strategies.

Bacterial stimuli such as CFA and CpG DNA had been shown to induce cross-priming when co-administered with a soluble protein antigen (Bennett *et al.*, 1997; Cho *et al.*, 2000). This research project examined the ability of a broader range of microbial stimuli to induce cross-priming. Of the stimuli tested, constituents of bacteria (LPS from some bacteria species, CpG DNA), of viruses (dsRNA, hemagglutinin from *Influenza* virus) and of fungi (mannan) were able to generate antigen-specific functional $CD8^+$ T cells responses when co-administered with a soluble protein antigen. Together with studies published during the course of this work (Le Bon *et al.*, 2003; Schulz *et al.*, 2005), this confirmed that the capacity to induce cross-priming was not restricted to one class of organisms.

It had been demonstrated that signals generated during a viral infection could authorise the occurrence of cross-priming, and this process was found to depend on IFN-α/βR signalling (Le Bon *et al.*, 2003). Therefore, as part of investigating the mechanisms by which cross-priming was induced, the contribution of IFN-α/βR signalling was examined. All microbial products tested that were able to induce cross-priming depended on IFN-α/βR signalling for licensing of cross-priming. In particular, poly(I:C), LPS from *E. coli*, LPS from *K. pneumoniae* K52 and K55, LPS from *N. meningitidis* and hemagglutinin from *Influenza* virus were almost completely dependent on IFN-α/β signalling. In the absence of IFN-α/βR, effector $CD8^+$ T cell

responses generated by cross-priming in response to mannan and CpG DNA were reduced approximately by three quarters and by half, respectively. This demonstrated that IFN-α/β signalling and other stimuli cooperated to induce cross-priming.

The use of anti-CD40 antibodies, co-injected with soluble OVA had shown that signalling through CD40 could be a licensing stimulus for cross-priming against non cell-associated exogenous antigens (Lefrancois *et al.*, 2000). However, results showed that mannan induced cross-priming independently of CD40 signalling, and experiments using CD40L- and CD40-deficient mice showed that licensing in response to CpG DNA is also not mediated by CD40 signalling (Sparwasser *et al.*, 2000; Van Uden *et al.*, 2001). While IL-12 was shown to contribute to induction of cross-priming by CpG DNA (Van Uden *et al.*, 2001), results showed that responses to mannan were independent of IL-12. Which additional signal(s) mannan triggers remains to be determined, but as it is the case for CpG DNA (Cho *et al.*, 2002), signalling through costimulatory molecules such as B7/CD28 may contribute to licensing of cross-priming.

Activation of IFN-α/βR signalling is therefore a major mechanism of licensing of cross-priming, which is triggered by signals from various microbial origins. Various factors may be involved in activation of IFN-α/βR signalling that is effective for licensing of cross-priming. For instance, the receptors stimulated by microbial stimuli, the intracellular pathways controlling IFN-α/β expression, the nature of IFN-α/β secreted and the cells producing them probably all influence whether IFN-α/β-dependent licensing of cross-priming can occur.

Induction of cross-priming by microbial stimuli initially depends on triggering of innate receptors that mediate the activation of intracellular signalling pathways, resulting in the expression of cross-priming licensing signals, including IFN-α/β. It has been shown that induction of cross-priming by poly(I:C) and CpG DNA is mediated through TLR3 and TLR9, respectively (Heit *et al.*, 2003; Schulz *et al.*, 2005). Results demonstrated that LPS from *E. coli* induced cross-priming through TLR4. LPS from *N. meningitidis* has been shown to require TLR4 for the activation of IFN-β (Zughaier *et al.*, 2005), therefore it is probable that induction of cross-priming by LPS from *N. meningitidis* is TLR4-dependent. The contribution of TLR4 in the response to LPS from *K. pneumoniae* K52 remains to be determined. Results

also showed that mannan required signalling through TLR4 to induce cross-priming. The triggering of TLR4-mediated responses by mannan has been demonstrated previously, and the effect of mannan was not sensitive to polymyxin B, showing that environmental LPS contamination was not responsible for mannan's properties (Tada *et al.*, 2002). The contribution of CD14 was not investigated, but CD14 may play an important role in activating TLR4-mediated induction of cross-priming by mannan. Indeed, CD14 has been shown to be involved in mannan- and other polysaccharide-induced TLR4-mediated responses (Shoham *et al.*, 2001; Flo *et al.*, 2002; Tada *et al.*, 2002). Furthermore, CD14 is required for TLR4/IRF3-mediated responses to VSV (Jiang *et al.*, 2005). Therefore, CD14 is able to activate IRF3-dependent IFN-α/β production through TLR4 in response to molecules other than LPS. In addition, since CD14 is involved in the differential response to smooth LPS (with O-antigen, constituted of carbohydrate repeating units) and rough LPS (no O-antigen), it is possible that the carbohydrate structure and/or conformation of mannan, VSV glycoprotein envelope (Stanley *et al.*, 1984), and *Influenza* HA enables interactions with CD14 and subsequent TLR4-dependent induction of cross-priming.

Therefore, TLR stimulation can mediate induction of cross-priming, probably through activation of pathways that control the expression of IFN-α/β. Further biochemical studies are however needed to define more precisely receptor/agonist interactions. Characterising the basic structural/conformational requirements for microbial component's stimulatory activity through TLR and receptor complexes would help to develop adjuvants with essential agonistic features.

While LPS from *E. coli* and mannan required TLR4 for induction of cross-priming, LPS from *K. pneumoniae* K55 did not. Polymannose LPS Kp K55, and LPS with similar structures (Ohta *et al.*, 1982; Kido *et al.*, 1985a; Ohta *et al.*, 1987), have previously been characterised as unusual LPSs, with potent adjuvant activities that were TLR4-independent (Ohta *et al.*, 1985). Among other properties, polymannose LPS greatly activates the complement system through the lectin pathway (Shibazaki *et al.*, 1999; Zhao *et al.*, 2002). Activation of the complement cascade generates intermediate products, such as C3 and chemotactic C5a (Ehlers, 2000; Guo *et al.*, 2005), that have been shown to play an important role in regulating adaptive immune responses (Carroll, 2004), including the generation of $CD8^+$ T cell responses (Kopf *et al.*, 2002; Suresh *et al.*, 2003; Kim *et al.*, 2004). It is therefore possible that LPS

Kp K55 is able to induce cross-priming in the absence of TLR4 through activation of complement, which may be verified by blocking TLR4 in C3-deficient mice for example. To characterise further the mechanisms of induction of cross-priming by stimuli such as polymannose LPS, the receptor(s) and signalling pathway(s) they activate would need to be defined.

Expression of IFN-α/β, which stimulate the IFN-α/βR signalling pathways, is controlled by nuclear transcription factors NF-κB and/or IRF (Akira *et al.*, 2004; Lohoff *et al.*, 2005), and activation of these factors has been characterised for a few but not all of the microbial constituents tested in this study.

Poly(I:C) and LPS from *E. coli* are known to induce the production of IFN-α/β through activation of IRF-3 (Hoebe *et al.*, 2003a; Hoebe *et al.*, 2003b; Oshiumi *et al.*, 2003a). dsRNA induces the production of IFN-α, including IFN-α4, and IFN-β (Juang *et al.*, 1998; Marie *et al.*, 1998), while LPS mainly induces the expression of IFN-β. To note, it is often inferred, based on the lack of detection of IFN-α mRNA or protein expression from LPS-stimulated bone-marrow derived DC or purified DC populations, that LPS does not induce the production of IFN-α (Hoshino *et al.*, 2002; Asselin-Paturel *et al.*, 2005; Honda *et al.*, 2005b). Results however showed that LPS from *E. coli* stimulated the secretion of IFN-α *in vivo* (up to 250 pg/mL), although to lower levels than poly(I:C) for instance (see 5.2.2). The effect of IFN-α4 and IFN-β is illustrated by the fact that IFN-α4 induces cross-priming against a co-administered soluble protein antigen (Le Bon *et al.*, 2003) and IFN-β shares this adjuvant property [Dr A. Le Bon, personal communication]. IFN-β and IFN-α4 have been shown to trigger an amplification loop by signalling through the IFN-α/βR, which induces the production of IRF7; IRF7 gets activated and induces the expression of IFN-α (Sato *et al.*, 1998; Sato *et al.*, 2000). Although exogenous poly(I:C) and dsRNA generated in the cytoplasm by viruses stimulate different receptors, both sources of dsRNA stimulate IRF-mediated expression of IFN-α/β through activation of the same kinases (TBK1 and IKKε) (Fitzgerald *et al.*, 2003a; Hemmi *et al.*, 2004; Kato *et al.*, 2005; Kawai *et al.*, 2005b). It has recently been shown that IRF7 is not required for IFN-β expression in response to NDV infection, while activation of IRF3 was essential (Honda *et al.*, 2005b). The same study confirmed that LPS induction of IFN-β is mediated by activation of IRF3 only. In addition, IFN-α and IFN-

β expression in response to poly(I:C) or LPS from *E. coli* was shown not to require signalling through the IFN-α/βR (Hoshino *et al.*, 2002; Hoebe *et al.*, 2003b).
CpG DNA in contrast induces the expression of IFN-α and IFN-β independently of IRF3, while activation of IRF7 is essential (Honda *et al.*, 2005a; Honda *et al.*, 2005b). It has also been shown that CpG DNA triggers an increase in IRF7 expression, and that expression of IRF7, IFN-α and IFN-β in response to CpG DNA is dependent on IFN-α/βR signalling (Asselin-Paturel *et al.*, 2005; Honda *et al.*, 2005b).

Therefore, although poly(I:C), LPS and CpG DNA all induce cross-priming through IFN-α/βR signalling, they trigger that event in different ways. Importantly, since responses to microbial stimuli can be controlled by feedback mechanisms, IFN-α/β production, as measured *in vitro* from purified cell populations or even *in vivo* from serum, is not a good correlate of downstream activation, as factors such as the nature of IFN-α/β secreted, the source of IFN-α/β, and therefore where IFN-α/β are produced, and kinetics of secretion most probably play a role in triggering signals such as cross-priming licensing. These factors may explain the fact that guanosine analogue R-848, although it induced the secretion of IFN-α systemically, was not able to induce cross-priming when co-administered with a soluble protein antigen. R-848 is known to induce IFN-α and IFN-β expression through activation of IRF7 and, as opposed to CpG DNA (Takaoka *et al.*, 2005), through IRF5 (Honda *et al.*, 2005b; Schoenemeyer *et al.*, 2005). As for CpG DNA, R-848 induction of IFN-α is dependent on IFN-α/βR signalling (Asselin-Paturel *et al.*, 2005). It is possible that R-848, as a result of IRF7 and IRF5 activation, induces the expression of IFN-α subtypes that are different from the ones produced in response to cross-priming-inducing stimuli such as poly(I:C), LPS and CpG DNA. There are fourteen different types of IFN-α in mice (van Pesch *et al.*, 2004), and R-848 may induce the production of combinations of IFN-α subtypes that trigger IFN-α/βR signalling pathways that do not support licensing of cross-priming. Indeed, it has been reported that individual IFN-α subtypes, and combinations of IFN-α subtypes and IFN-β, activate different IFN-α/βR signalling pathways and induce the expression of different patterns of genes (Bartlett *et al.*, 2002; Cull *et al.*, 2002; Harle *et al.*, 2002;

Cull *et al.*, 2003). The characterisation of IFN-α subtype secretion profiles is presently limited by the lack of specific detection reagents.

IFN-α production *in vivo* in response to both CpG DNA and R-848 is mainly performed by plasmacytoid DC (pDC) (Asselin-Paturel *et al.*, 2005). However, responses to R-848 differ from responses to CpG DNA and notably R-848 induces different pDC migration patterns (Asselin-Paturel *et al.*, 2005). Indeed, pDC cluster to different areas at different times in secondary lymphoid organs in response to R-848 or CpG DNA. pDC play an important role in DC-induced $CD8^+$ T cell responses (Yoneyama *et al.*, 2005). Therefore, it is possible that, in response to R-848, pDC are not localised appropriately, or at the right time, for receiving positive feedback signals and secreting the IFN-α/β that would license DC to induce cross-priming.

It is known that DC are the APC that mediate cross-priming (Jung *et al.*, 2002) and current knowledge indicates that $CD8\alpha^+$ DC are the particular subsets that perform cross-priming (den Haan *et al.*, 2000). While expression of TLR3, TLR4 and TLR9 enables $CD8\alpha^+$ DC to respond directly to poly(I:C), LPS and CpG DNA, respectively, $CD8\alpha^+$ DC do not express TLR7 and therefore cannot respond directly to R-848 (Edwards *et al.*, 2003). It is thus probable that poly(I:C) and LPS can directly trigger a cross-priming licensing state in $CD8\alpha^+$ DC, which as many cell types constitutively express IRF3 (Au *et al.*, 1995), through TLR3/IRF3/IRF7- and TLR4/IRF3/IRF7-mediated IFN-α/β activation pathways, respectively. CpG DNA does not directly induce production of IFN-α/β from $CD8\alpha^+$ DC, probably because these DC, as opposed to pDC, do not constitutively express IRF7, and CpG DNA does not activate IRF3. However, CpG can directly stimulate pDC to produce IFN-α/β, which in turn may induce the expression of IRF7 in $CD8\alpha^+$ DC. Direct activation of $CD8\alpha^+$ DC by CpG DNA, which can induce the production of cytokines as well as costimulatory molecules (Asselin-Paturel *et al.*, 2005; Honda *et al.*, 2005b), in combination/synergy with activation by pDC-produced IFN-α/β may induce a cross-priming licensing state in $CD8\alpha^+$ DC. This may be illustrated by the fact that induction of cross-priming by CpG DNA does not solely depend on IFN-α/β signalling pathways but depend on additional factors such as costimulatory molecules and IL-12 signalling pathways (Cho *et al.*, 2002). In contrast, R-848 is not able to induce cross-priming possibly because not only it cannot directly stimulate

$CD8\alpha^+$ DC, but also because R-848-activated pDC may not be able to induce any cross-priming licensing state in $CD8\alpha^+$ DC through IFN-α/βR signalling, possibly due to the production of inefficient combinations of IFN-α subtypes and IFN-β, and/or due to a lack of pDC/$CD8\alpha^+$ DC co-localisation at the right time.

Therefore, in order to characterise the role of IFN-α/β signalling in the induction of cross-priming, the IFN-α/β-producing and -responding cells, as well as the nature, timing and location of IFN-α and IFN-β production, would need to be defined.
The downstream effects of IFN-α/β signalling pathways in licensing of cross-priming remain to be determined, as it is still not known what IFN-α/β-induced factors control cross-priming of $CD8^+$ T cells by DC, whether it involves soluble or membrane proteins, or both.

The generation of antigen-specific antibody responses contributes to increasing the efficiency of the immune responses. Antibodies can rapidly activate the complement cascade through the classical pathway, which leads to pathogen destruction by the membrane attack complex. Potent antibody responses are characterised by the secretion of antibody from different classes and isotypes, since different types of antibodies have different functions that cooperate to prevent infection and to eliminate pathogens. Isotype switching controls the production of different types of antibodies and it requires appropriate help from antigen-specific $CD4^+$ T cells, through cytokine secretion and costimulatory molecule activation. Results demonstrated that antigen-specific $CD4^+$ T cells primed in the presence of mannan and LPS from *K. pneumoniae* K52 and K55 differentiated into cytokine-producing cells. $CD4^+$ T cell effector function corroborated the fact that the same microbial stimuli enhanced antigen-specific IgG responses and promoted isotype switching. $CD4^+$ T cells specifically differentiated into Th1 cells, as they produced IFN-γ but no IL-4. IFN-γ supports switching to IgG isotypes, and antigen-specific IgG2a responses in particular were enhanced by LPSs and mannan; the high-mannose viral glycoprotein HA also promotes antigen-specific IgG2a responses [Dr A. Le Bon, personal communication]. IgG2a antibodies play an important role in pathogen killing as they trigger CMC and ADCC against infected cells, and the latter mechanism is supported by IFN-γ. Although the effect of poly(I:C), LPS from *E. coli* and CpG DNA on priming of $CD4^+$ T cell responses was not examined, the fact that

these stimuli enhanced antigen-specific IgG responses and promoted isotype switching, to IgG2a especially, is a strong indication that antigen-specific $CD4^+$ T cell help was generated. The signals produced in response to poly(I:C), LPSs, mannan and CpG DNA were potent enough to stimulate the generation of memory antibody responses, including of the IgG2a isotype, probably, among other mechanisms, through the priming of effective $CD4^+$ T cell help.

Therefore, detection of poly(I:C), LPS from *E. coli*, LPS from *K. pneumoniae* K52 and K55, mannan and CpG DNA induces signals that enhance antigen-specific antibody responses and promote IgG switching to isotypes that are most appropriate for the killing of intracellular pathogens. The mechanisms mediating the effect of microbial stimuli on antigen-specific antibody responses were not investigated. However, IFN-α/β have been shown to enhance antibody responses against a soluble protein antigen and to promote isotype switching, and IFN-α/βR signalling mediated the adjuvant effect of poly(I:C) (Le Bon *et al.*, 2001). Therefore it is probable that in response to LPSs, mannan and CpG DNA, IFN-α/βR signalling mediates both the enhancement of antibody responses and induction of cross-priming.

This investigation confirmed and extended results from previous studies demonstrating that the generation of functional $CD8^+$ T cell responses against an exogenously administered antigen is possible in the presence of stimuli that induce IFN-α/β signalling-dependent cross-priming. However, efficient vaccines against intracellular pathogens would not only require the stimulation of primary antigen-specific $CD8^+$ T cells responses, but also the development of protective immunity through the induction of immunological memory. Although results demonstrated that in response to poly(I:C), LPS from *E. coli*, LPS from K. *pneumoniae* K52 and K55 and CpG DNA, B cells received appropriate help from $CD4^+$ T cells to develop antigen-specific memory responses, it may have not been the case for $CD8^+$ T cells.

Therefore, the ability of microbial stimuli to promote the generation of memory $CD8^+$ T cells, capable of effector functions upon rechallenge, against an exogenous antigen needs to be assessed. Studying the effect of diverse microbial stimuli on the induction of memory $CD8^+$ T cells would contribute to defining what signals activate the immune system in such a way that a memory program is imprinted into $CD8^+$ T cells during priming. Indeed, although under intense investigation, mechanisms of

and factors affecting immunological memory are insufficiently defined at present. Integration of knowledge in IFN-α/β-dependent cross-priming mechanisms and development of immunological memory would be required to design safe effective vaccines to prevent or combat a number of serious infections.

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Appendix

Publication arising from this work

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Special Feature

Shaping of adaptive immune responses to soluble proteins by TLR agonists: A role for IFN-α/β

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